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Microbial biofilms of varied composition and thickness develop on all surfaces in contact with aqueous environments, a process known as biofouling. Microorganisms are organisms that cannot be seen as individuals with the unaided human eye. Microorganisms use polysaccharides to cement themselves to surfaces, grow, reproduce, and produce more extracellular polymers. Biofilms contain immobilized cells embedded in an absorptive and porous organic polymer matrix of microbial origin. As a result, biofilms consist of a large percentage of adsorbed and entrapped materials, including solutes, heavy metals, and inorganic particles, such as silt and clay, in addition to the cellular constituents.

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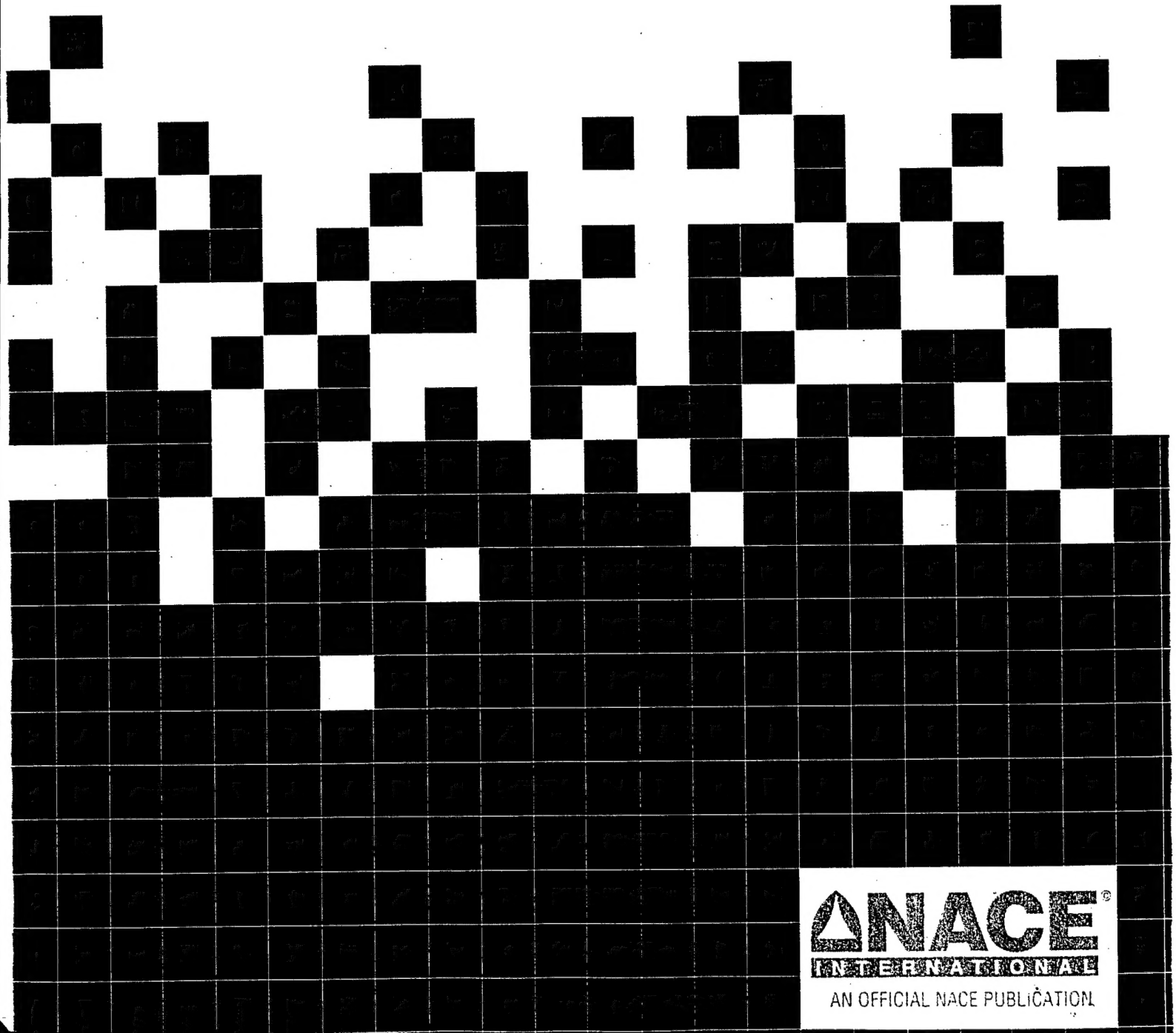
Corrosion Testing Made Easy

Microbiologically Influenced Corrosion

Brenda J. Little, Patricia A. Wagner, and Florian Mansfeld

B.C. Syrett, Series Editor



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Corrosion Testing Made Easy *Volume 5*

Microbiologically Influenced Corrosion

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Preface to the Series

The *Corrosion Testing Made Easy* series was conceived in 1983 by members of the NACE Publications Committee in recognition of the need for easy-to-follow guidelines on specific types of corrosion testing. While many test procedures are defined in NACE and industry standards and some textbooks, it was noted that none of these sources was written with the beginner in mind; all assumed some prior experience with corrosion testing and familiarity with corrosion terminology.

This situation inevitably leads to a need for the entry-level technician to be trained initially by a more experienced experimentalist. The cost of such training can be quite high, especially if a highly paid professional scientist or engineer must do the training. Furthermore, because of demands on their time, the more experienced experimentalist may not always be available to give timely advice. Consequently, there seemed to be an urgent need for a series of books that could guide an entry-level technician through a corrosion test procedure with little or no assistance. These books would make no assumption about prior knowledge or expertise. They would describe the possible difficulties in performing the experiment and would indicate how to correct mistakes if they were made. The books would each contain an unusually large number of illustrations on the grounds that "one good picture is worth a thousand words." Hence, the *Corrosion Testing Made Easy* series was created.

Each author for the series was chosen with great care. All authors are technically very competent, all have had many years of hands-on experience in performing corrosion testing, and, perhaps even more important, they have a reputation for clear, concise writing.

Most of the books in the series can be used as step-by-step guides to performing specific types of corrosion tests, e.g., stress corrosion tests or electrochemical tests. However, the introductory volume, *The Basics*, describes techniques and equipment that are common to most, if not all, corrosion tests. It provides a glossary that relates to corrosion, and it contains some basic principles of corrosion and electrochemical theory. Although the practical aspects of corrosion testing are emphasized in all volumes in the series, theoretical aspects are introduced when they can help the experimentalist perform a test with greater ease.

More than a dozen volumes are planned for publication in the next few years. While they are introduced primarily for entry-level-technicians, it is clear that these books may be also useful for more experienced scientists and engineers. For instance, engineers skilled in the art of performing fatigue tests may use the *Corrosion Testing Made Easy* series to obtain practical advice about extending their knowledge of corrosion fatigue tests. Or an experienced biologist may need help in performing a seawater corrosion test as part of a larger study on marine toxicology. Whoever uses the series, one thing is certain—the volumes are unique and fill an important gap in scientific literature.

Barry C. Syrett

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This work was supported by the Office of Naval Research, Program Element 0601153N, and the Defense Research Sciences Program, NRL Contribution Number NRL/BA/7333-95–0005. The authors acknowledge the assistance of Kevin Hart, NRL Code 7333; and Maria Banker, Darlene Jorns, and Maryellen Turcotte, NRL Code 7032, in manuscript preparation; and suggestions of NACE International reviewers.

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Contents

CAUTION

The test methods described in this publication may involve hazardous materials, operations, and equipment. This publication does not purport to address all of the safety problems associated with the test methods. It is the responsibility of whoever uses these test methods to consult and establish appropriate safety and health practices and to determine the applicability of regulatory limitations before use.

CHAPTER 1

Introduction

Microbial biofilms of varied composition and thickness develop on all surfaces in contact with aqueous environments, a process known as biofouling. Microorganisms are organisms that cannot be seen as individuals with the unaided human eye. Microorganisms use polysaccharides to cement themselves to surfaces, grow, reproduce, and produce more extracellular polymers. Biofilms contain immobilized cells embedded in an absorptive and porous organic polymer matrix of microbial origin. As a result, biofilms consist of a large percentage of adsorbed and entrapped materials, including solutes, heavy metals, and inorganic particles, such as silt and clay, in addition to the cellular constituents.

Biofilm formation and metallic corrosion have traditionally been evaluated as separate, independent processes that occur simultaneously or sequentially on metal surfaces. However, the role of microorganisms in the corrosion of metals has received increased attention in recent years as chemical processing, oil and gas, and power generation industries have acknowledged the occurrence and prevalence of microbiologically influenced corrosion (MIC) in operating systems. MIC has been documented for metals exposed to seawater, freshwater, demineralized water, process chemicals, foodstuffs, soils, fuels, human plasma, and sewage.

Testing for MIC can determine (1) whether a particular metal/microbe combination will result in an unacceptable level of corrosion or (2) whether microbes are responsible for specific failures. *There are no definitive tests for MIC.* Instead, several types of circumstantial evidence contribute to the conclusion that MIC will take place or has taken place. MIC does not result in a specific type of localized attack. MIC can result in pitting, crevice corrosion, under-deposit corrosion, dealloying/selective leaching, or enhanced erosion corrosion. It is helpful to know the numbers and types of microorganisms that can be linked to an apparent corrosion mechanism, their spatial distribution, and their relationship to localized corrosion even though there is no direct correlation between the number of microorganisms and the likelihood of MIC.

MIC detection and verification techniques involve inspection of corroded materials, test specimens, or corrosion deposits to identify specific types and numbers of bacteria (e.g., sulfate-reducing bacteria [SRB] and metal-depositing bacteria), or corrosive byproducts, such as chlorides, acids, or sulfides.

Four types of evidence are used to verify MIC: metallurgical, microbiological, chemical, and electrochemical. In the following sections, the reader will be introduced to the fundamentals of biofilm formation, cellular constituents of biofilms, mechanisms for MIC and, most importantly, tests that can be used in the laboratory and/or the field to determine the likelihood and extent of MIC.

Biofilm Formation

Microorganisms are classified as either eucaryotes or procaryotes. Eucaryotes have a true nucleus and include algae, fungi, and protozoa. Procaryotes lack a true nucleus and include bacteria. Protozoa may be amoeboid, flagellated, or ciliated. They are predators of bacteria and algae, removing them from biofilms.

Algae are unicellular photosynthetic organisms that sometimes are motile and colored. They are found in a wide range of environments — from freshwater to concentrated brines, pH from 5.5 to 9.0, and temperatures from below 0°C to 40°C (32°F to 104°F). In the presence of light, algae produce oxygen (photosynthesis) that can accumulate within biofilms. In the absence of light, algae consume oxygen (respiration) and reverse the process. Diatoms are microalgae that have silicon-containing frustules and often are the most conspicuous constituents within biofilms (Figure 1).

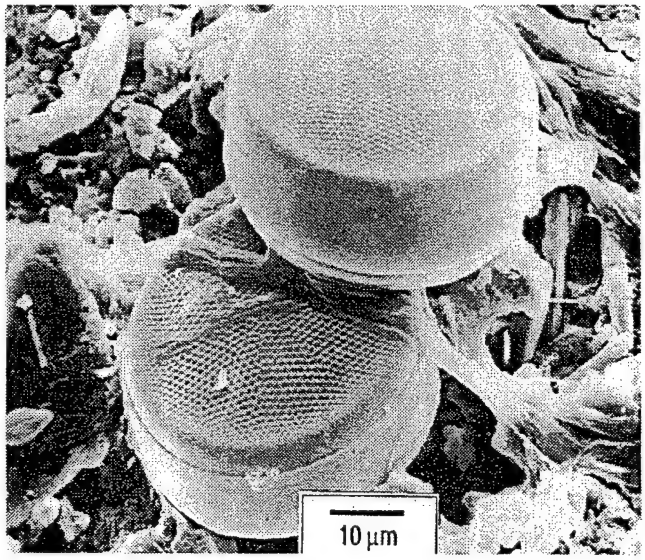


FIGURE 1 - Scanning electron micrograph of diatoms from a marine biofilm.

Fungi are nonphotosynthetic and have a vegetative structure known as a mycelium that is the outgrowth of a single reproductive cell or spore. Neither spores nor mycelia are capable of movement. A mycelium can grow to an indefinite length so fungi often reach macroscopic dimensions. Although soil is the most common habitat of fungi, many fungal groups are aquatic. Fungi assimilate organic material and produce organic acids, including oxalic, lactic, acetic, and citric. Yeasts are fungi that multiply by forming buds.

Bacteria have received the most attention for their presence and activities within biofilms. Bacteria may be spherical, rod-shaped, filamentous, or helical (Figure 2). They may occur individually, but they tend to form colonies within the biofilm, reproducing by binary fission or cell division.

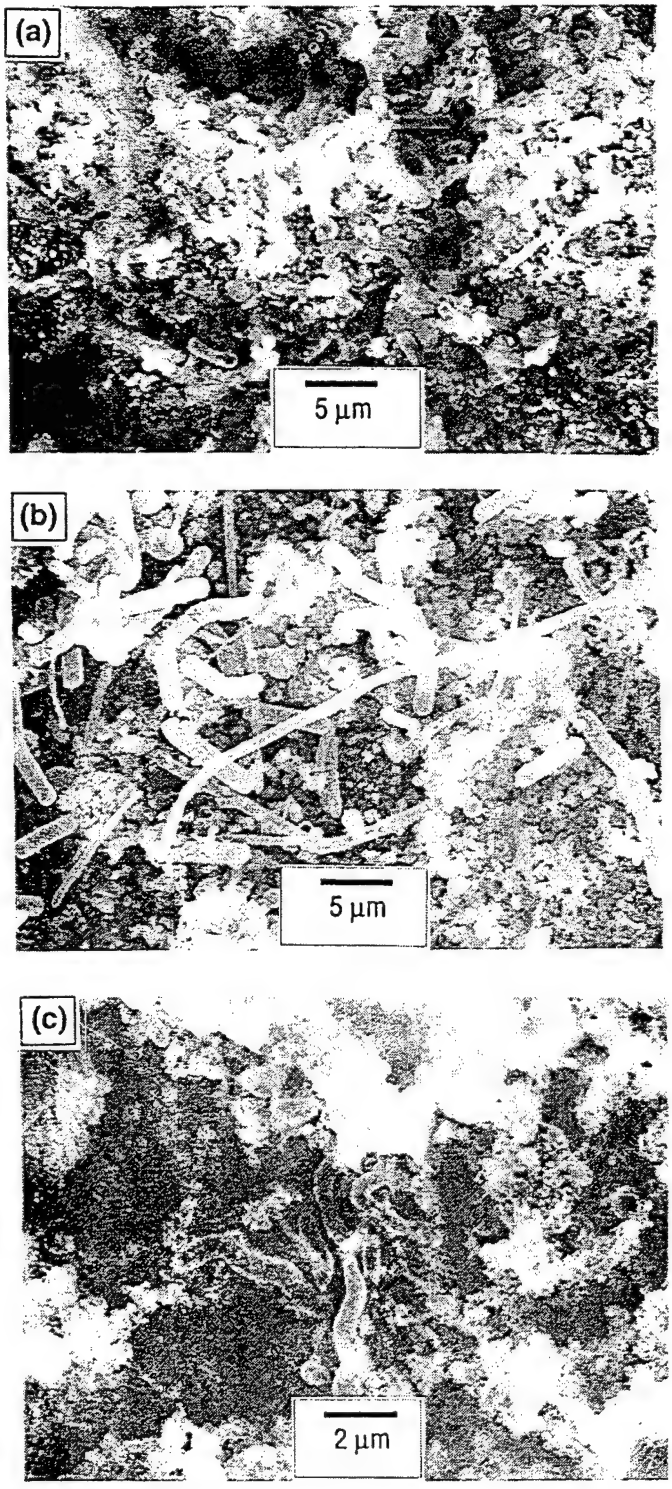


FIGURE 2 - Environmental scanning electron micrographs of cells within a biofilm (a) spherical, (b) rod shaped and filamentous, and (c) helical.

Filamentous bacteria usually range in length from 1-10 µm and are about 0.2 µm wide, although some filaments may be several hundred µm long. Bacteria can tolerate a wide range of environmental conditions, including temperatures from less than 0°C to 99+°C (32°F to 210+°F) and pH from 0 to 12. Bacteria can be grouped according to requirements for oxygen and sources of energy:

- Obligate aerobes require oxygen for survival and growth.
- Microaerophilic bacteria require low oxygen concentration.
- Facultative anaerobic bacteria prefer aerobic environments, but can live under anaerobic conditions.
- Obligate anaerobic microorganisms cannot tolerate oxygen for growth and survival.
- Obligate anaerobic bacteria are routinely isolated from oxygenated environments associated with particles, inside crevices, and most importantly, in association with other bacteria that effectively remove oxygen from the immediate vicinity of the anaerobe.

Bacteria also can be grouped according to their nutritional requirements. Heterotrophic bacteria derive energy from a wide range of organic molecules. As a group, heterotrophic bacteria can assimilate almost any available carbon molecule, from simple alcohols and sugars to complex polymers. Autotrophic bacteria oxidize inorganic compounds, elements, or ions (e.g., NH_3, NO^-_2, H_2, CO_2, Fe^{+2}, Mn^{+2}) as sources of energy.

It should be emphasized that the nutritional requirements for laboratory culture of a single bacterial species might be quite specific, but in a mixed population, the nutritional needs of one bacterial group may be provided by other members of the community. Biofilms provide protective environments for bacteria, and in most cases, allow different types of bacteria to flourish within strata of the biofilm (Figure 3). Bacteria within biofilms act cooperatively to produce conditions more favorable for growth of each species. Bacteria nearest an oxygenated fluid phase are provided with complex nutrients and oxygen. These bacteria use oxygen, break down carbon sources, and produce simple polymers and fatty acids. Bacteria deeper within the biofilm use these waste products as nutrients that are metabolized to fatty acids, carbon dioxide, and hydrogen. Successive stages of degradation depend on the chemistry of the liquid phase and bacterial species. For example,

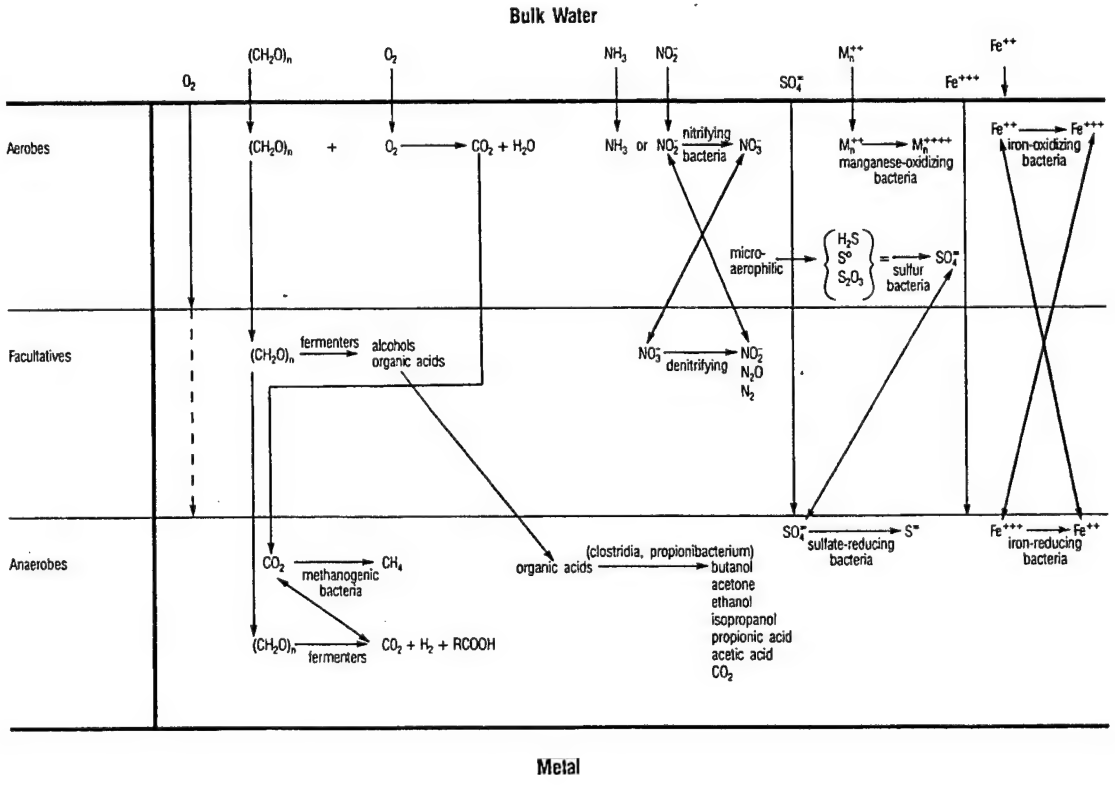


FIGURE 3 - Strata within a typical biofilm and possible reactions within the strata.

acetogenic bacteria convert nonfermentable compounds to carbon dioxide or acetic acid and hydrogen, or both. Other microorganisms consume acetate and hydrogen. When sulfates are present, sulfate-reducing bacteria (SRB) predominate because of the ability of some species to produce the enzyme hydrogenase that reduces the hydrogen concentration to such a low level that other bacteria cannot compete. Hydrogenase breaks hydrogen molecules down to constituent protons and electrons and transfers energy to the organism through a series of organometallic molecules in the electron transport system.

Substratum, the surface to which microorganisms attach, plays a major role in biofilm processes during the early stages of biofilm accumulation and may influence the rate of cell accumulation as well as distribution of the cells. Electrolyte concentration, pH, and inorganic ions influence settlement. The presence of hydrated oxide and hydroxide passivating films on metal surfaces provides bacteria with sites for firm attachment. Similarly, spalling or sloughing of corrosion products results in detachment of biofilms associated with corrosion products. Microbial colonization of a substratum increases with increasing surface roughness. Welds, for example, provide increased numbers of sites for colonization compared to smooth pipe surfaces.

Energy derived from organic carbon drives heterotrophic microbial growth within biofilms. Generally, increasing the total organic carbon (TOC) increases the assimilable carbon substrate available to the biofilm. At high organic loadings, substrate flux into the biofilm will reach a constant value as a result of one of these:

- The growth rate of the microbial population in the biofilm reaches a maximum,
- The thickness of the biofilm exceeds the penetration depth of the substrate into the biofilm, or
- The electron acceptor or another nutrient becomes nutrient-limiting.

Figure 4 is a composite of all processes contributing to biofilm accumulation. Organic carbon is present in all natural and processed waters, but its concentration can vary widely. For example, TOC in seawater off the coast of Hawaii is 1 g m^{-3}; distilled water, 1–2 g m^{-3}; Gulf of Mexico coastal water, 10 g m^{-3}; oilfield-produced water, 150 g m^{-3}; and untreated sewage, 200 g m^{-3}.

Carbon is not always the growth-limiting nutrient. Phosphorus and nitrogen may be limiting in some aquatic systems. An electrolyte with a carbon to nitrogen ratio (C/N) equal to or greater than 7 is considered nitrogen-limited for microbial growth. Cells growing in such a medium tend to produce copious amounts of extracellular polymer.

Hydrodynamic shear stress, related to flow, influences transport, transfer, and reaction rates within the biofilm as well as detachment. Bulk water temperature influences the rate of most chemical and biochemical reaction processes as well as transport processes within biofilms. Biofouling is generally more of a problem in the summer months because higher temperatures increase the rate of biological processes.

MIC Mechanisms

Microorganisms within biofilms are capable of maintaining an environment radically different from that of the bulk medium in terms of pH and dissolved oxygen as well as organic and inorganic species. In some cases, these interfacial conditions could not be maintained in the bulk medium at atmospheric pressure and room temperature. As a result, MIC can lead to the production of corrosion products that would not be predicted from abiotic experiments and thermodynamic analyses.

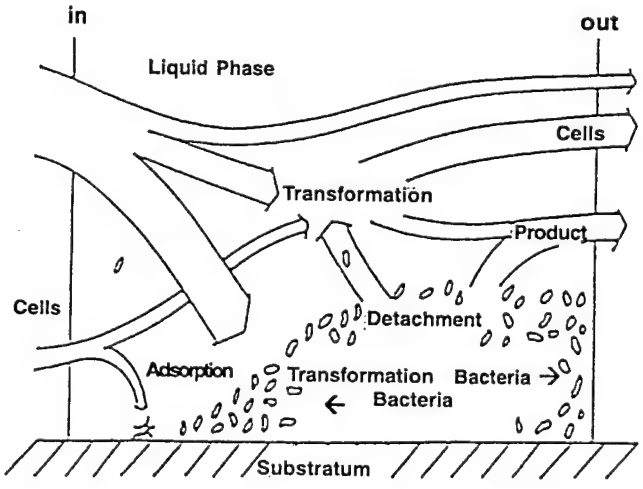


FIGURE 4 - A composite of processes contributing to biofilm accumulation.[1]
(W.G. Characklis, "Biofilm Processes," in *Biofilms*. ©1990 Reprinted by permission of John Wiley & Sons, Inc.)

Concentration Cells

In aqueous media, macromolecules adsorb on all surfaces prior to colonization by microorganisms. Adsorption of organic material from the aqueous phase alters the interfacial surface energy of the solid as well as the corrosion potential (E_{corr}) of metal surfaces. The physical presence of microbial cells on the surface, in addition to their metabolic activities, modifies electrochemical processes. Adsorbed cells grow, reproduce, and form colonies that are physical anomalies on metal surfaces, resulting in local anodes and cathodes. Under aerobic conditions, areas under respiring colonies become anodic and surrounding areas become cathodic (Figure 5).

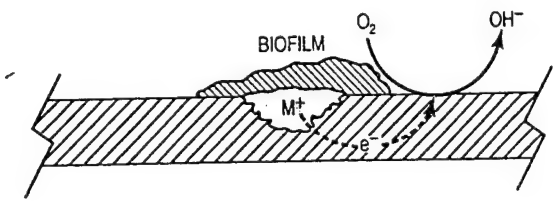


FIGURE 5 - Biofilm formation produces a localized anodic site.

A mature biofilm that covers the entire surface prevents diffusion of oxygen to cathodic sites and diffusion of aggressive anions, such as chloride, to anodic sites. Outward diffusion of metabolites and corrosion products also is impeded. If areas within biofilms become anaerobic, i.e., if the aerobic respiration rate is greater than the oxygen diffusion rate, a change in the cathodic mechanism occurs.

Reactions within Biofilms

Metabolic processes within biofilms affect corrosion. It is traditional to discuss microorganisms within biofilms as aerobic or anaerobic, and by individual species. However, microorganisms form synergistic communities that conduct combined processes that individual species cannot. Furthermore, a single microorganism simultaneously can affect corrosion via several mechanisms. Cell death or lysis within a well-developed biofilm does not necessarily mean a cessation of the influence on electrochemical processes. For example, pitting corrosion continues under deposits of iron-depositing bacteria independent of the biochemical activity of the bacteria.

Algae and photosynthetic bacteria use light to produce oxygen that can accumulate within a biofilm. During dark periods they respire, converting oxygen to carbon dioxide. Over the normal range of pH values encountered in seawater, oxygen availability has a greater influence on corrosion than pH changes. Oxygen can depolarize the cathodic reaction, leading to increased corrosion rates. Localized respiration/photosynthesis can lead to differential aeration cells and localized anodes and cathodes.

When bacteria metabolize nutrients, the organisms must dispose of the electrons produced by metabolic processes. In aerobic microorganisms, the terminal electron acceptor is usually oxygen, which is converted to water. In anaerobic environments, SRB use the sulfate ion as the terminal electron acceptor, producing hydrogen sulfide. SRB have been the focus of many investigations involving MIC. The following electrochemical reactions involving SRB have been suggested for MIC of iron:

$$4Fe \rightarrow 4Fe^{+2} + 8e^- \quad \text{(anodic reaction)} \qquad (1)$$

$$8H_2O \rightarrow 8H^+ + 8OH^- \quad \text{(water dissociation)} \qquad (2)$$

$$8H^+ + 8e^- \rightarrow 8H \quad \text{(adsorbed) (cathodic reaction)} \qquad (3)$$

$$SO_4^{-2} + 8H \rightarrow S^{-2} + 4H_2O \quad \text{(bacterial consumption)} \qquad (4)$$

$$Fe^{+2} + S^{-2} \rightarrow FeS \quad \text{(corrosion products)} \qquad (5)$$

The overall process has been called cathodic depolarization based on the theory that some SRB could remove the atomic hydrogen accumulated at the cathode. Removal of cathodic hydrogen forces more iron to be dissolved at the anode. Some strains of SRB also can stimulate corrosion by cathodic depolarization induced by microbiologically produced FeS. The impact of oxygen on obligate anaerobic SRB has been examined, and the highest corrosion rates are observed during periods of aeration.

Most heterotrophic bacteria secrete organic acids during fermentation of organic substrates. The types and amounts of acids produced depend on the kind of microorganisms and available substrate molecules. Organic acids may force a shift in the tendency for corrosion to occur as measured by the potential shift between anodes and cathodes. The impact of acidic metabolites is intensified when they are trapped at the biofilm/metal interface. Acetic acid from *Clostridium aceticum* and sulfuric acid from *Thiobacillus thioxidans*

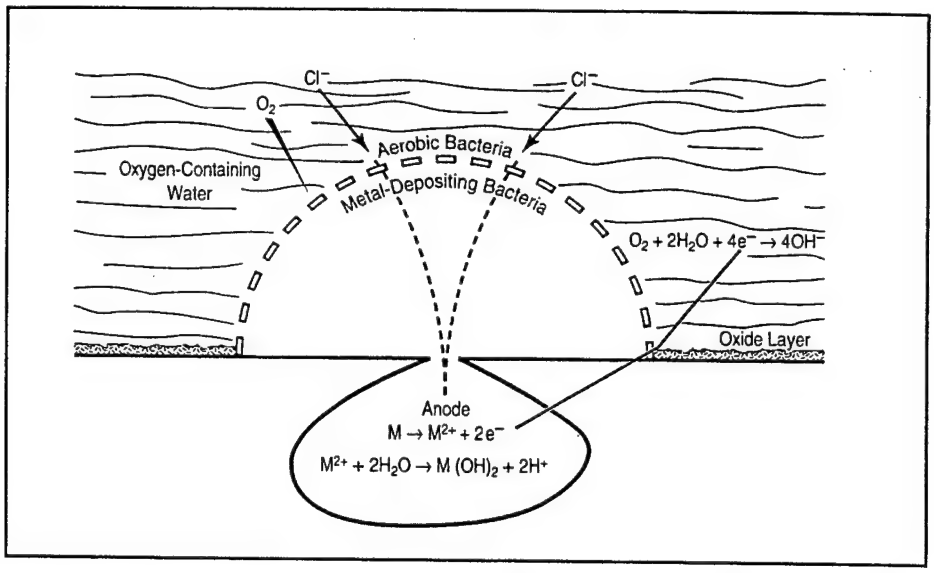


FIGURE 6 - Possible reactions under tubercles created by metal-depositing bacteria.

are obvious contributors to corrosion. Organic acids produced during oxidation of organic compounds can promote the electrochemical oxidation of a variety of metals by removing or preventing formation of an oxide film. The pH under algal fouling varies with photosynthesis/respiration. Daily pH changes of up to two units have been recorded with values above 10 during photosynthesis. In decaying algal cultures, pH values as low as 1.8 have been measured.

Many organisms produce ammonia (NH_3) from the metabolism of amino acids or the reduction of nitrite or nitrate. Corrosion failures in copper alloys due to the presence and activities of ammonia-producing bacteria have been documented. In solution, ammonia ionizes to the ammonium ion that can react with some metals and alloys to cause corrosion.

In recent years, the role of metal-depositing bacteria in MIC has been stressed. Metal-depositing microorganisms can catalyze oxidation of metals, accumulate metal oxides as precipitates, or derive energy by oxidizing metals. Iron-depositing bacteria usually cited as causing MIC are *Gallionella*, *Sphaerotilus*, *Crenothrix*, and *Leptothrix*. These organisms oxidize ferrous ions to ferric ions or manganous ions to manganic ions to obtain energy. Metal-depositing organisms create environments conducive to corrosion. Dense deposits of cells and metal ions create oxygen concentration cells that effectively exclude oxygen immediately under the deposit.

Under-deposit corrosion is very important because it initiates a series of events that are individually or collectively extremely corrosive. In an oxygenated environment, the area immediately under the deposit, deprived of oxygen, becomes a relatively small anode compared to the large, surrounding oxygenated cathode. The metal will form metal cations at anodic sites. If the cathodic and anodic sites are separated from one another, the pH at the anode decreases while pH at the cathode increases. If the metal hydroxide is the thermodynamically stable phase in the solution, metal ions will be hydrolyzed by water with the formation of H^+ ions. The pH in the pit depends on the metallurgy of the alloy (Table 1.1). Pit interiors in stainless steels containing chromium can have pH values close to 1.0.

Chloride (Cl^-) ions from the electrolyte will migrate to the anode to neutralize any buildup of charge, forming extremely corrosive metal chlorides (Figure 6). Under these circumstances, pitting involves the conventional

TABLE 1.1
SPECIFIC HYDROLYSIS REACTIONS

HYDROLYSIS REACTION	EQUILIBRIUM pH
$Fe^{2+} + 2H_2O \rightleftharpoons Fe(OH)_2 + 2H^+$	$pH = 6.64 - 1/2 \log a_{Fe^{2+}}$
$Cr^{3+} + 3H_2O \rightleftharpoons Cr(OH)_3 + 3H^+$	$pH = 1.53 - 1/3 \log a_{Cr^{3+}}$
$Ni^{2+} + 2H_2O \rightleftharpoons Ni(OH)_2 + 2H^+$	$pH = 6.5 - 1/2 \log a_{Ni^{2+}}$
$Mn^{2+} + 2H_2O \rightleftharpoons Mn(OH)_2 + 2H^+$	$pH = 1.53 - 1/3 \log a_{Mn^{2+}}$

features of differential aeration, a large cathode to small anode surface area ratio, and development of acidity and metallic chlorides.

The following roles have been proposed for microorganisms in hydrogen embrittlement of metals:

1. Production of molecular hydrogen during fermentation, which may be dissociated into atomic hydrogen and absorbed into metals;
2. Production of hydrogen ions via organic or mineral acids, which may be reduced to form hydrogen atoms at cathodic sites;
3. Production of hydrogen sulfide, which may stimulate the absorption of atomic hydrogen into metals by inhibiting its recombination into hydrogen molecules; and
4. Destabilization of metal oxide films, allowing easier absorption of hydrogen by the underlying metal.

Microorganisms produce exopolymers and form gel matrices on metal surfaces that are central to the structural integrity of microbial films. Gels can have numerous effects on interfacial processes, including:

1. Immobilizing water at the biofilm/metal interface;
2. Binding metal ions (e.g., copper, manganese, chromium or iron) from the electrolyte or from the substratum;
3. Trapping corrosion products at the interface;
4. Decreasing diffusion;
5. Preventing the effectiveness of corrosion inhibitors and/or biocides.

In general, exopolymers are acidic and contain functional groups that bind metals. Accumulation of metal ions from the aqueous phase or from the substratum can result in metal concentration cells and increased corrosion.

Inactivation of Corrosion Inhibitor

Aliphatic amines, nitrites, and chromates that are used as corrosion inhibitors can be degraded by microorganisms, decreasing the effectiveness of the compounds and increasing the microbial populations. Organic corrosion inhibitors, including aliphatic amines and thiorerea, can be used in bacterial metabolism.

Problems have been reported in cooling water systems in which chromate levels could not be maintained because of bacterial reduction of Cr (VI) to Cr (III).

Case Histories

An illustrated reference manual that extensively describes case histories of MIC in industrial and marine environments has been compiled by G. Kobrin and is available from NACE.[3] The color photos are recommended for MIC identification.

Ferrous Metals

A large number of MIC-influenced failures have been reported for mild steel piping and equipment in chemical/pharmaceutical plants, oil refining industry ground distribution, fossil fuel power plants, steel-rolling mills, and the power industry (Figures 7-10). Acid-producing bacteria were the primary cause of MIC of carbon steels.

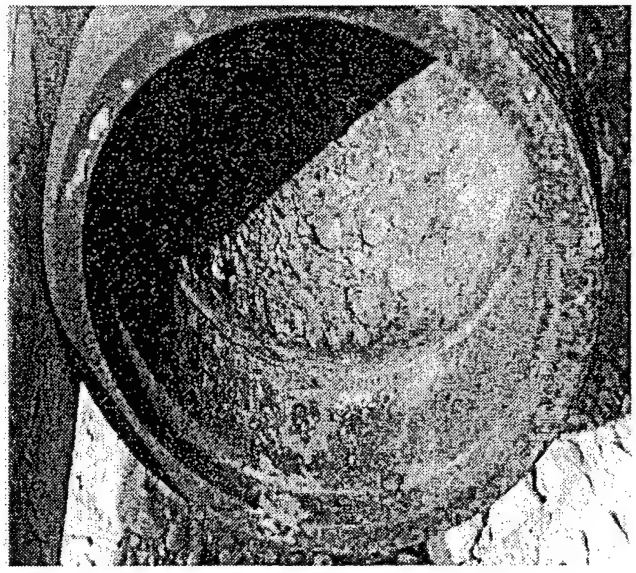


FIGURE 7 - Steel groundwater supply pipeline showing internal pitting due to MIC.[4] (Reprinted with permission of The Institute of Materials.)

Unprotected mild steel undergoes uniform corrosion in seawater at a rate of 0.1 to 0.6 mm y^{-1}. In general, however, steels are not exposed to seawater in practical applications without cathodic protection, protective coatings, or a combination of both. Holidays or breaks in protective coatings can be sites for growth of microorganisms, and breakdown products of polymer coatings may provide nutrients for acid-producing bacteria (Figures 9, 10).

FIGURE 8 - Mild steel rising main showing general encrustation, blistering, and localized corrosion and perforation due to MIC.[4] (Reprinted with permission of The Institute of Materials.)

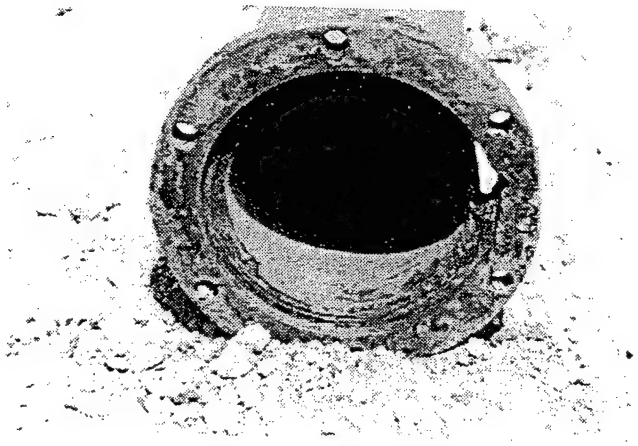


FIGURE 10 - Flanged end of coal tar epoxy-coated mild steel rising main. MIC concentrated around weld zone of flange section.[4] (Reprinted with permission of The Institute of Materials.)

Iron- and manganese-depositing bacteria have been identified with localized corrosion of stainless steel alloys 304 and 316. Pitting is almost always concentrated at weld seams, heat-affected zones (Figures 11-13), and gasketed joints (Figures 14, 15). Additional examples are provided in Figures 16-20.

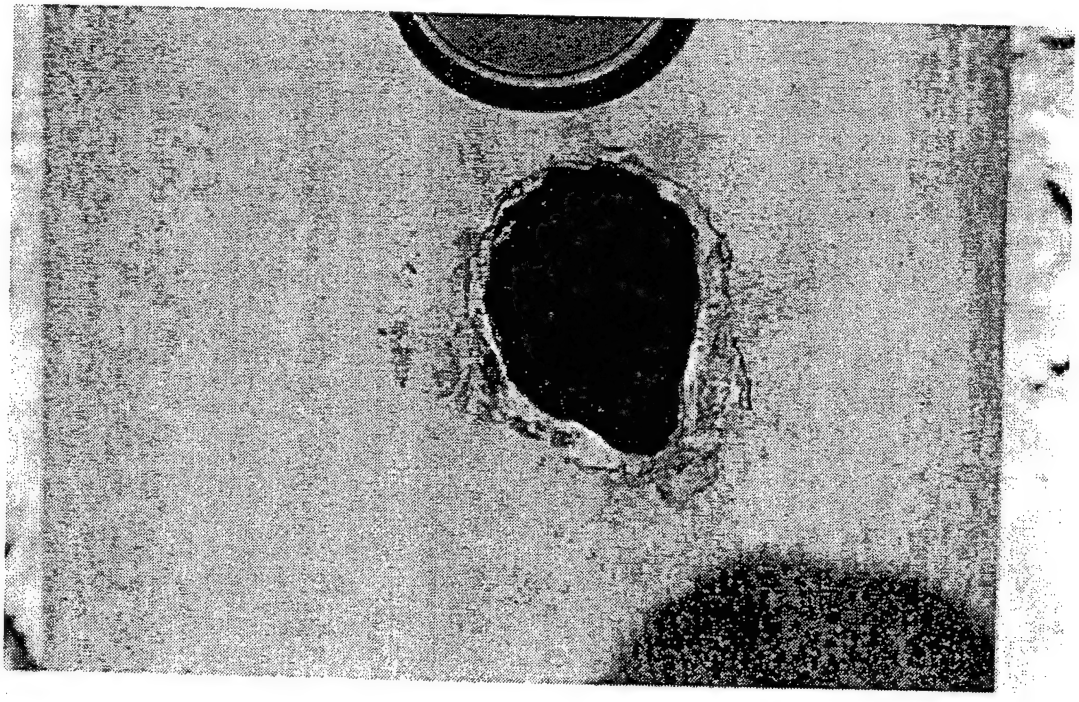


FIGURE 9 (right) - Coal tar epoxy coated mild steel pump column with perforation due to MIC probably at point of coating damage.[4] (Reprinted with permission of The Institute of Materials.)

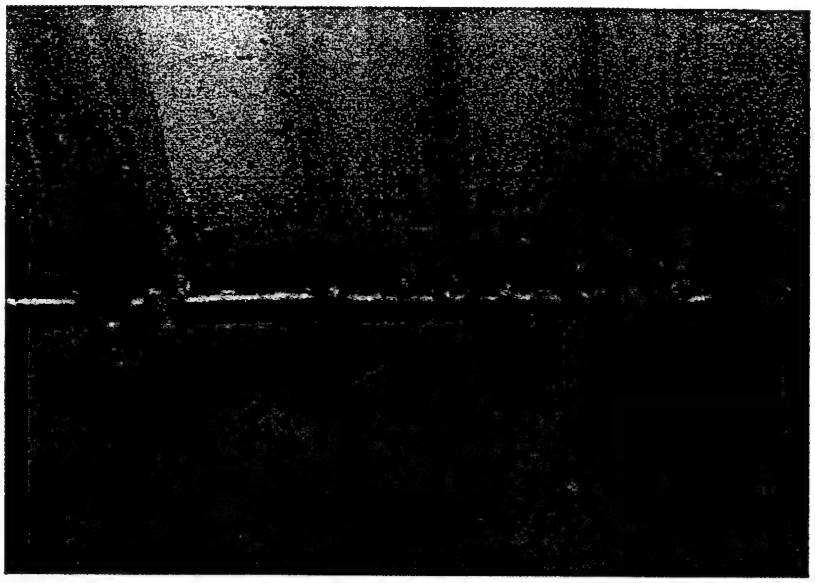


FIGURE 11 - Pits and deposits at weld seam in 316L stainless steel after exposure to potable water.[3]

FIGURE 12 - Cross section through a pit in a weld seam of a 304 stainless steel tank under a deposit. Note small opening (arrow) on the surface as the entrance in a large round subsurface cavity.[3]

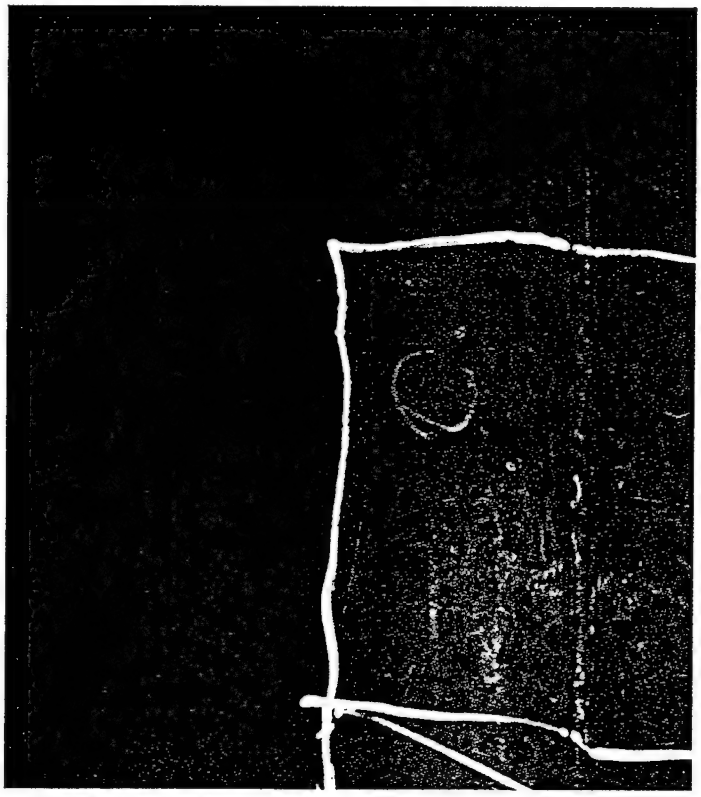


Figure 13 (right) - Pitting associated with MIC at the heat-affected zone of a vertical seam weld inside the 304 stainless steel tank.[3]

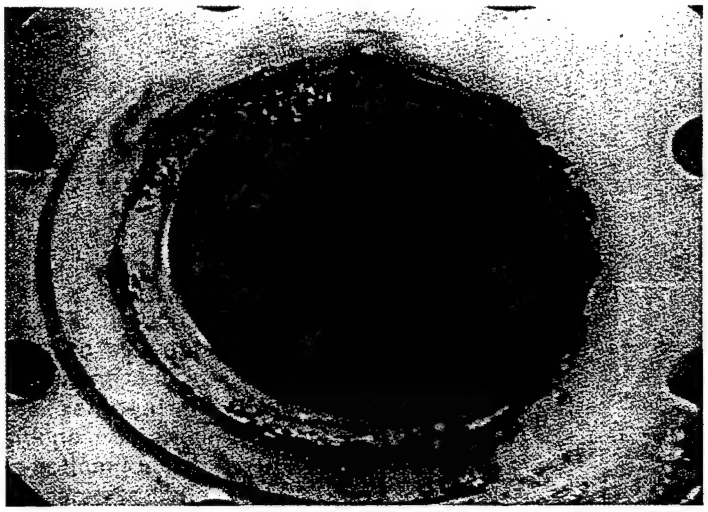


FIGURE 14 - 304 stainless steel flange showing a deposit associated with MIC. The flange had been covered with an asbestos gasket and a stainless steel blind flange.[3]

FIGURE 15 - Closeup of flange in Figure 14 after cleaning shows open "gouging" corrosion directly under deposits.[3]

FIGURE 16 - Section of 304 stainless steel air distribution piping showing bacterial deposits, especially on underside.[3]

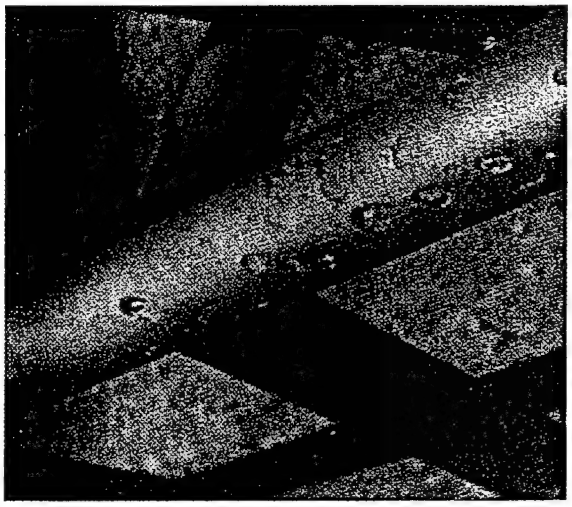


FIGURE 17 - (right) Underside of pipe shown in Figure 16 after blast cleaning.[3]

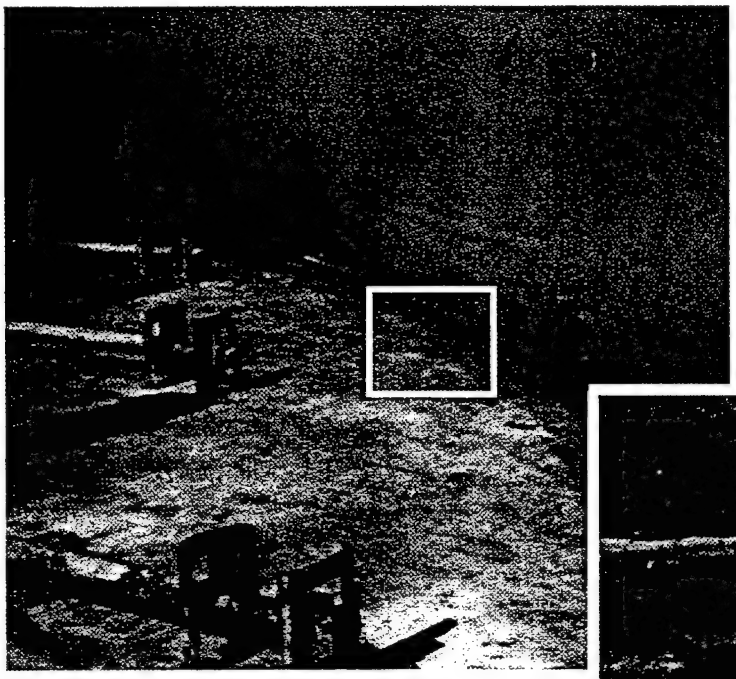


FIGURE 18 (right) - Portion of waste treatment tank after removal of sludge. Sidewall-to-bottom junction appeared sound at this stage.[3]

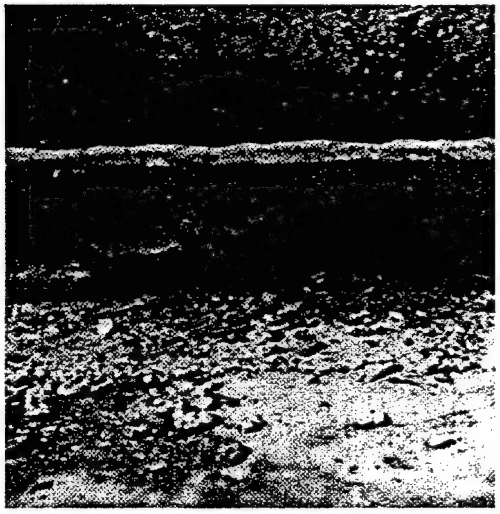


FIGURE 19 (right) - Closeup of sidewall-to-bottom junction in Figure 18 after removal of soft material, showing penetration.[3]

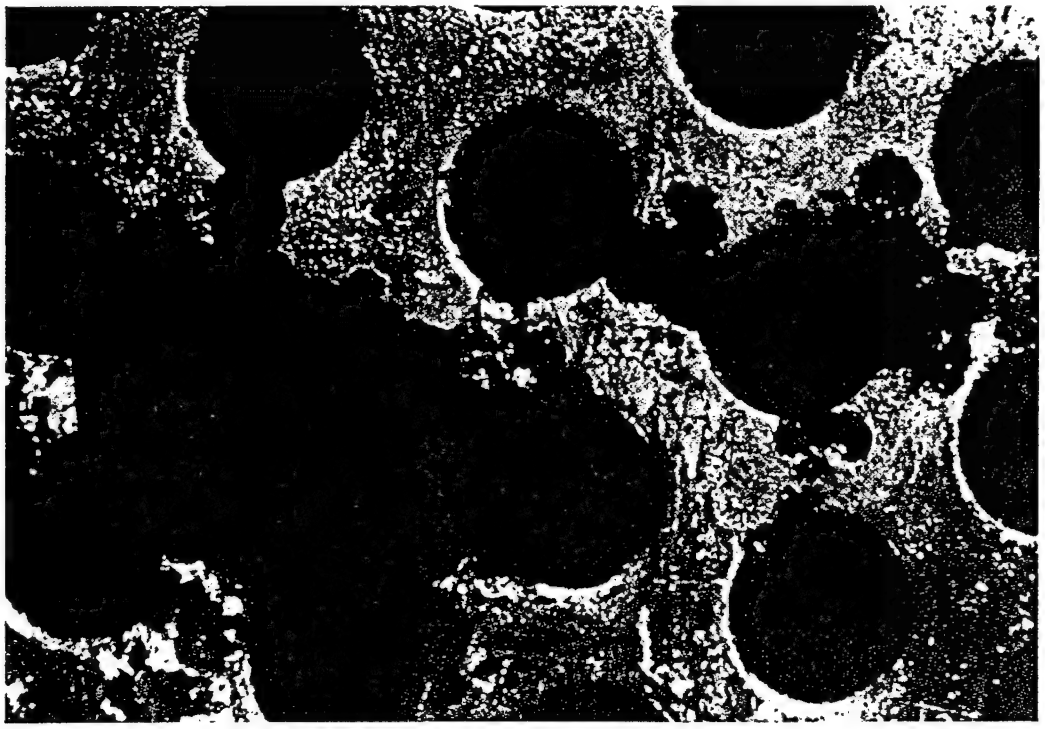


FIGURE 20 - Corrosion of a 304 stainless steel perforated screen, attributed to SRB. [3]

A change of material does not always cure a MIC problem. For example, an upgrade from carbon steel to stainless steel has led to different and sometimes more severe MIC problems. The importance of the microstructure of welds, the ferrite content, and sensitization in the failure of austenitic steel welds has been evaluated. Lack of sensitization does not ensure protection from MIC. Many of these failures in stainless steels have been attributed to hydrotest procedures that introduce naturally occurring water into piping systems to test the integrity of the welds. After hydrotesting, systems may not be effectively drained, leaving stagnant or low-velocity conditions for weeks or months at temperatures favorable to the establishment and growth of microbiological colonies, which can produce localized environments, causing rapid attack on welds. In one case, alloy 304 (UNS S30400) pipeline designed for a 30-year lifetime failed when it was put into freshwater service about 15 months after hydrotesting.

The corrosion resistance of stainless steels is due to the formation of a thin passive film. Passivity can break down under the following, usually localized, conditions:

- Dilute and concentrated HCl, HBr, and HF, and salts that hydrolyze these acids;
- Oxidizing chlorides, such as $FeCl_3$, $CuCl_2$, or NaOCl;
- Non-oxidizing halogenated waters, such as brines and seawater, except for brief exposures;
- Organic acids, including oxalic, lactic, and formic acids.

As described in the section on mechanisms, formation of oxidizing chlorides is accelerated by tubercles formed by metal-depositing bacteria.

A persistent problem with the use of stainless steels is susceptibility to crevice corrosion. The crevice area, usually small compared to the large cathodic surrounding area, will become the anode and the crevice electrolyte will become acidic. Diffusion of chloride ions into the crevice will lead to a corrosive solution of heavy metal chlorides of low pH. A literature survey of MIC of stainless steels shows that in many cases the corrosive action of the microorganisms is due to crevice formation.

A common method of corrosion protection for steel exposed to atmospheric conditions or natural waters is the application of a solid zinc layer, a process known as galvanizing. Zinc acts as a sacrificial anode and protects the steel exposed in pinholes and scratches in the zinc layer. MIC has been identified in cooling towers associated with commercial air conditioning systems (Figure 21). Biological analyses of the material from within the pits showed the presence of both aerobic sulfur-oxidizing bacteria (SOB) as well as SRB. The SOB created acidic conditions, dissolving the zinc coating locally without leaving the often observed mounds of corrosion products.

Delamination of the zinc coating under deposits of iron-depositing bacteria has been documented (Figure 22 a, b).

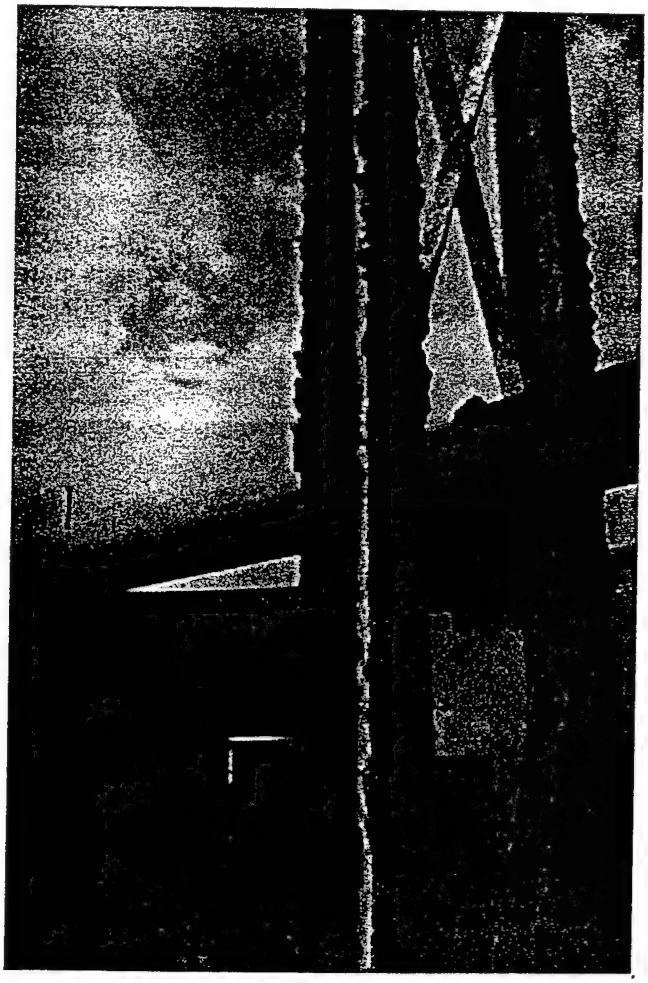


FIGURE 21 - Galvanized steel supports showing bacteria and corrosion product. Severe corrosion was found under deposits.[3]

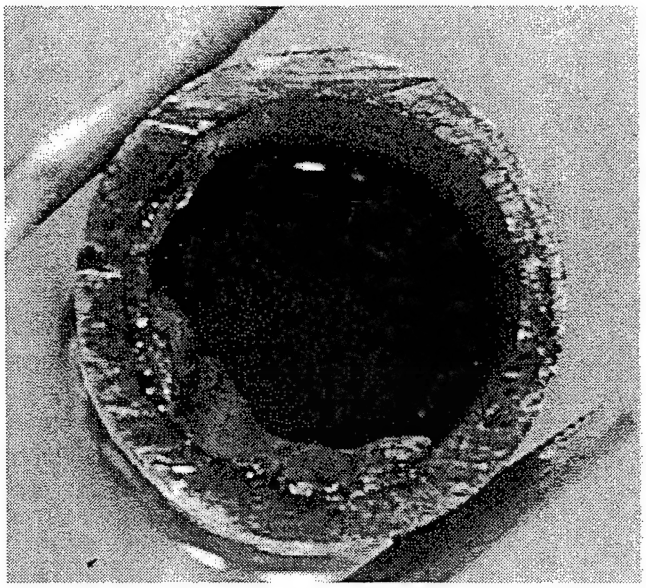


FIGURE 22(a) - Iron-depositing bacteria deposits in galvanized freshwater piping.

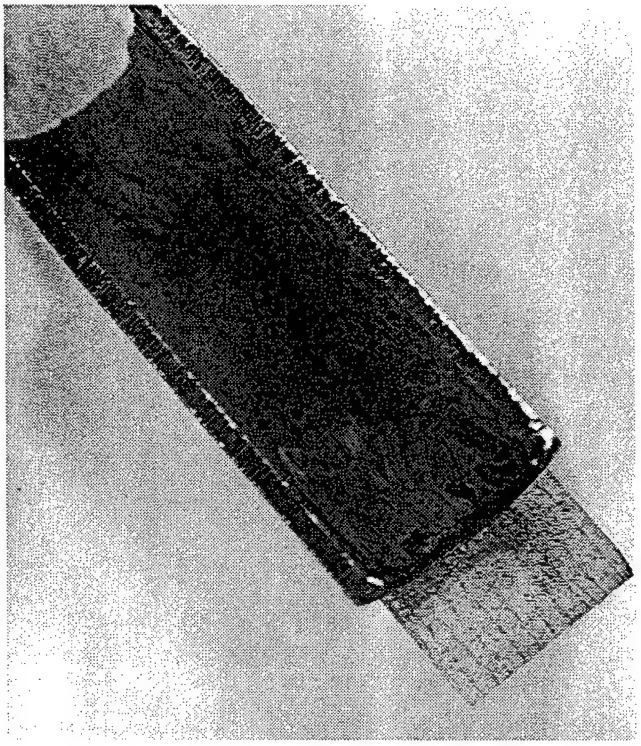


FIGURE 22(b) - Through-wall pitting from Figure 22(a).

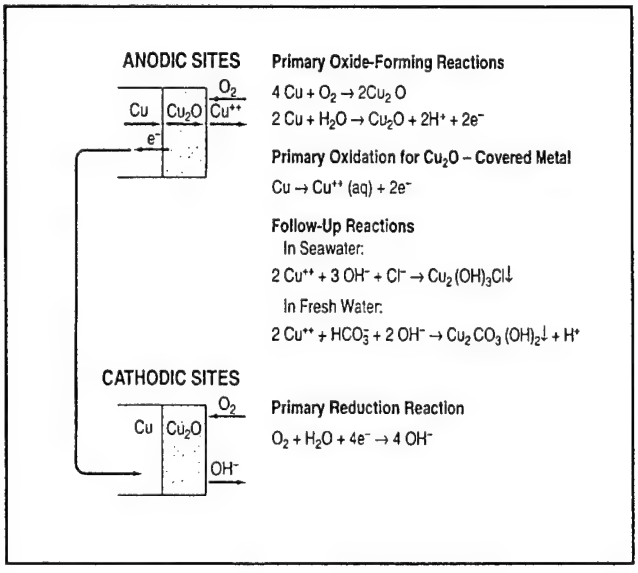
Copper and Copper Alloys

Copper alloys frequently are used for seawater piping systems and heat exchangers because of good corrosion resistance combined with mechanical workability, excellent electrical and thermal conductivity, ease of soldering and brazing, and a resistance to macrofouling. In oxygenated seawater, a film of cuprous oxide, Cu_2O, forms on the metal. Copper ions and electrons pass through the film. Copper ions dissolve and precipitate as $Cu_2(OH)_3Cl$ independent of alloy chemistry.

Figure 23 presents a set of reactions consistent with observed corrosion behavior for copper alloys. Alloying nickel and iron into copper alters the corrosion product and improves corrosion resistance.

Copper alloys prevent or retard the settlement of macrofouling species, such as barnacles and mussels. However, bacteria, microalgae, protozoa, and their cellular exudates form a slime layer on copper-containing surfaces. Copper fouls at a slower rate than stainless steel and glass surfaces, and the microflora on copper surfaces is less diverse than that found on steel and glass exposed under identical conditions.

Copper alloys are vulnerable to MIC. MIC has been



FGURE 23 - Typical cathodic and anodic reactions oin copper alloys in oxygenated seawater and freshwater.

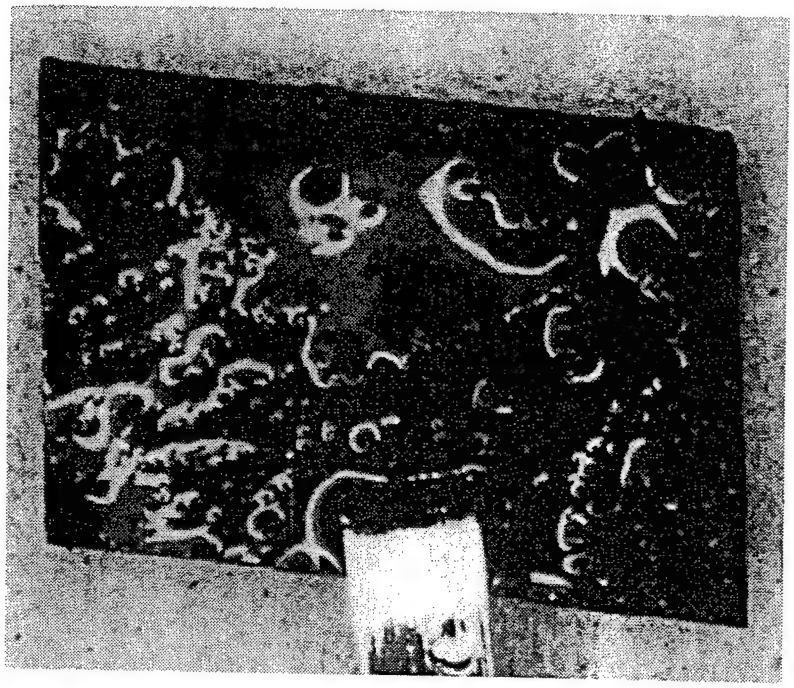


FIGURE 24 - Black sulfide film on 99Cu foil after four months exposure to SRB.

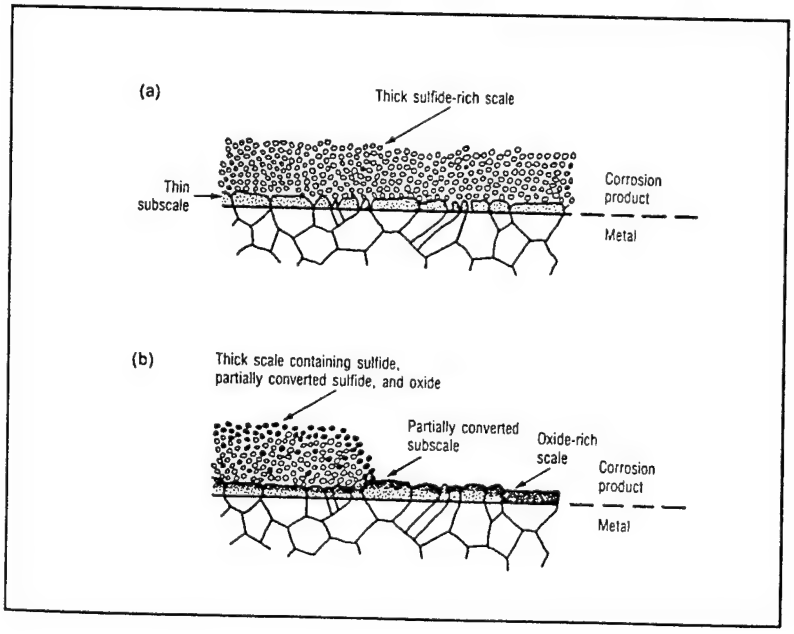


FIGURE 25 - Schematics of (a) thick sulfide rich scale on copper alloy and (b) disruption of sulfide film.[5]
(Reprinted from B.C. Syrett, "The Mechanism of Accelerated Corrosion of Copper-Nickel Alloys in Sulfide-Polluted Seawater," *Corrosion Science*, Vol. 21 (3) 1981, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK)

reported for 99Cu, 90Cu:10Ni, 70Cu:30Ni, admiralty and aluminum brass, and aluminum bronze. Differential aeration, selective leaching, under-deposit corrosion, and cathodic depolarization have been reported as mechanisms for MIC of copper alloys.

These chemicals produced by microorganisms accelerate localized attack: CO_2; H_2S; NH_3; organic and inorganic acids; metabolites that act as depolarizers; and sulfur compounds, such as mercaptans, sulfides, and disulfides. Extracellular polymers secreted by bacteria also can play a role in the corrosion of coper alloys by binding copper ions from the sustratum, producing copper concentration cells.

The impact of sulfides on the corrosion of copper alloys has received considerable attention. Pitting and stress corrosion cracking of copper alloys has been reported in polluted seawater containing sulfides. 90Cu:10Ni suffered accelerated corrosion attack in seawater containing 0.01 ppm waterborne sulfide after one-day exposure. A porous layer of cuprous sulfide forms in the presence of sulfide ions. Copper ions migrate through the layer, react with more sulfide, and produce a thick, black scale (Figure 24). In the presence of turbulence, the loosely adherent sulfide film is removed, exposing a fresh copper surface to react with the sulfide ions. For these reasons, erosion or turbulence-induced corrosion and sulfide attack of copper alloys cannot easily be decoupled. In the presence of oxygen, the possible corrosion reactions in a copper sulfide system are extremely complex because of the large number of stable copper sulfides, differing electrical conductivities, and catalytic effects. Transformations between sulfides, or of sulfides to oxides, result in changes in volume that weaken the attachment scale and oxide subscale, leading to spalling. Bared areas repassivate, forming cuprous oxide (Figure 25). Sulfide attack and dealloying of nickel from a 90Cu::10Ni seawater fire protection system are presented in Figure 26(a-d).

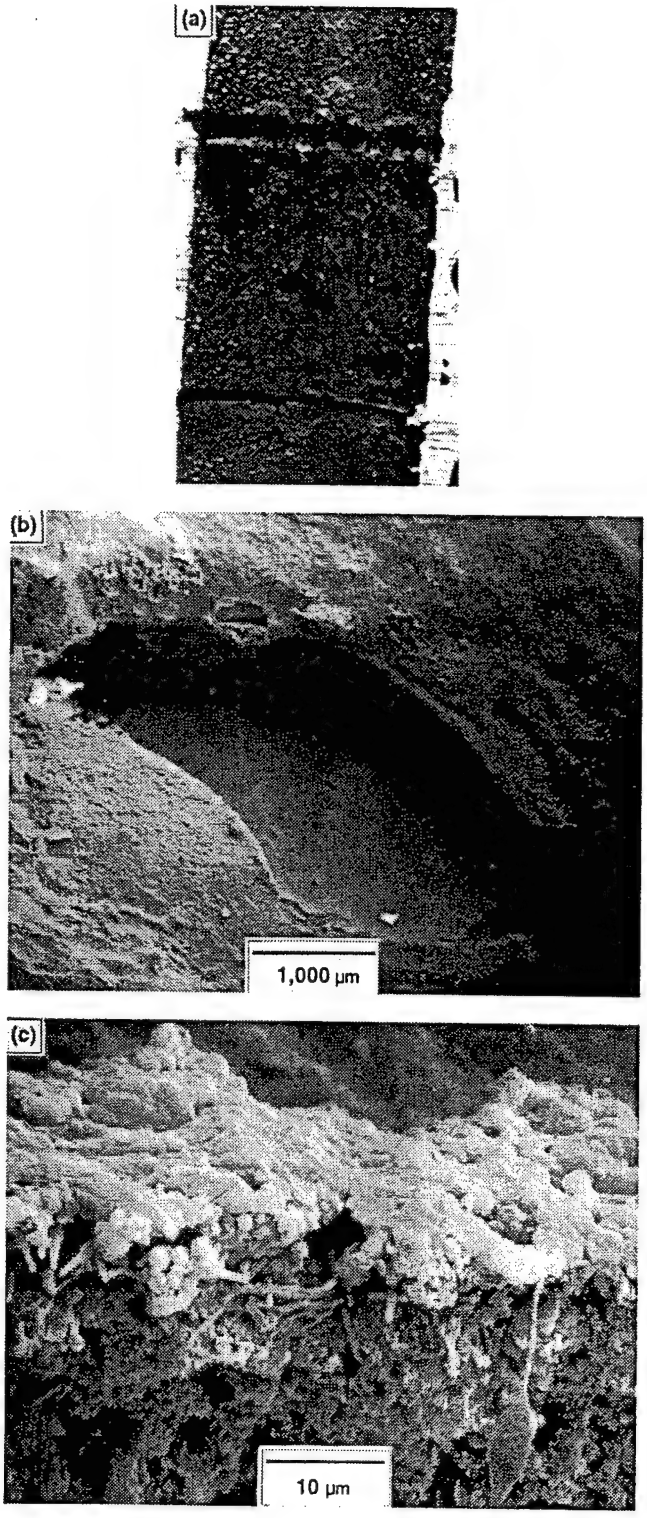


FIGURE 26 - Pitting in copper-nickel seawater piping system after one year in service: (a) pitted area, (b) pit, and (c) cross section of pit showing bacteria on top and residual spongy material.

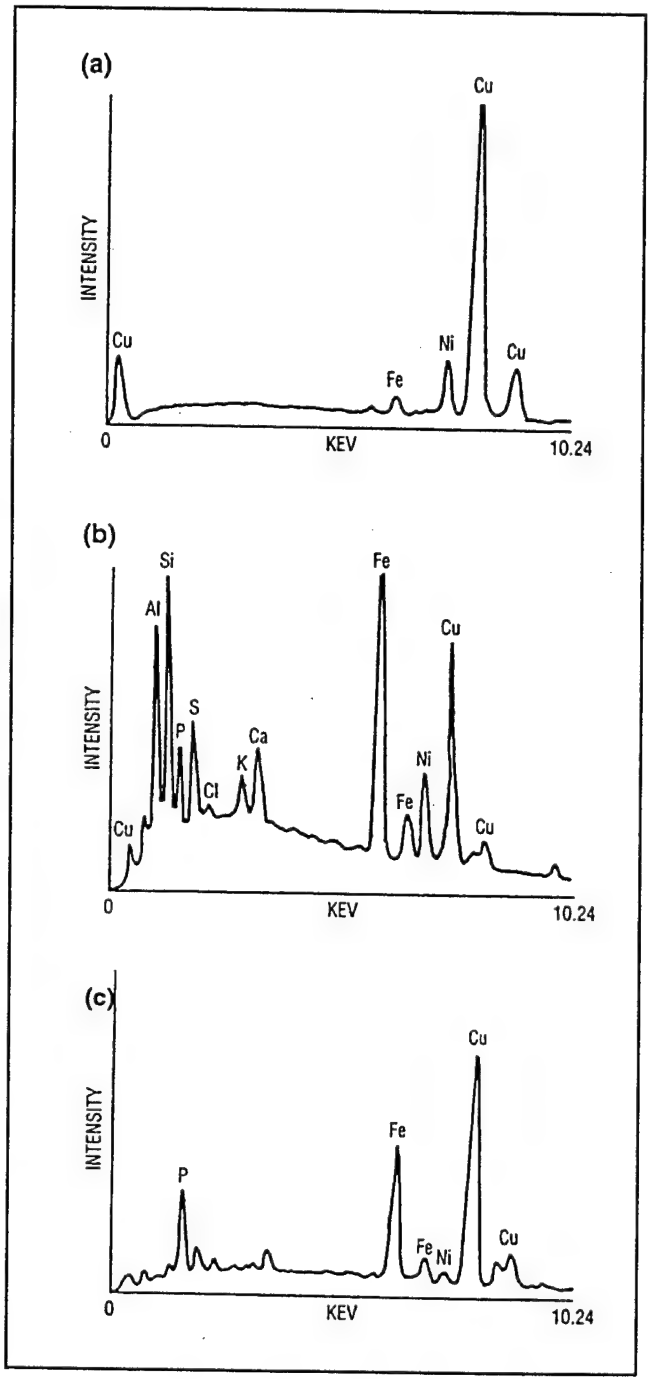


FIGURE 26(d) - Energy dispersive spectroscopy (EDS) spectra: (a) clean copper alloy before exposure; (b) pit showing accumulation of aluminum, silicon, phosphorus, sulfur, calcium, and elevated amounts of iron and nickel; (c) spongy material beneath bacteria showing accumulation of phosphorus, enrichment of iron, and depletion of nickel in the pit base.

Nickel Alloys

Nickel alloys generally are regarded as corrosion-resistant. Alloys, such as type 400 (UNS N04400) containing 66.5% nickel, 31.5% copper, and 1.25% iron have been used extensively in highly aerated, fast-moving seawater environments, such as evaporators, heat exchanger pumps and valves, diffusers for steam nozzles in steam ejectors, and turbine blades. In flowing seawater, the reported corrosion rate is typically 0.012 mm y^{-1}. A passive film similar in structure to that observed on pure nickel is formed on nickel-copper alloys having more than 30% nickel. Alloys containing less than this amount of nickel behave like copper. The formation of the protective film on nickel is aided by the presence of iron, aluminum, and silicon. In high-velocity, unpolluted seawater, nickel alloys are superior to predominantly copper alloys because the protective surface film remains intact under highly turbulent and erosive conditions.

Type 400 has a marked tendency for initiating pitting in chloride-containing environments where the passive film can be disturbed. Under stagnant conditions chlorides can penetrate the passive film and cause pitting attack. Sulfides can cause either a modification of the oxide layer, as described for copper, or breakdown of the oxide film of nickel-based alloys. Pit initiation and propagation depend on depth of exposure, temperature, and presence of surface deposits.

Like copper-containing alloys, type 400 is susceptible to under-deposit corrosion and oxygen concentration cells (Figure 27). Pits develop under deposits of SRB, and nickel is selectively dealloyed (Figure 28). No evidence of MIC in nickel-chromium or nickel-molybdenum alloys has been reported.

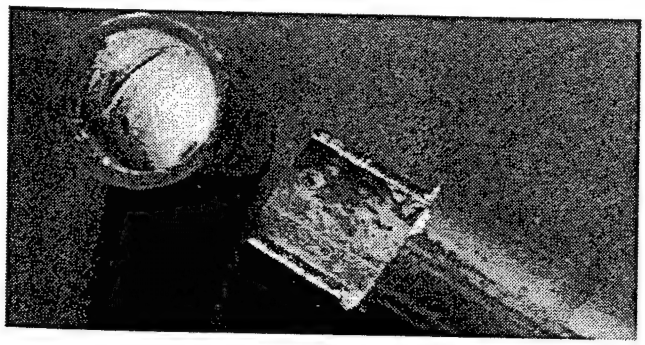


FIGURE 27- Localized corrosion of a type 400 heat exchanger tube.[3]

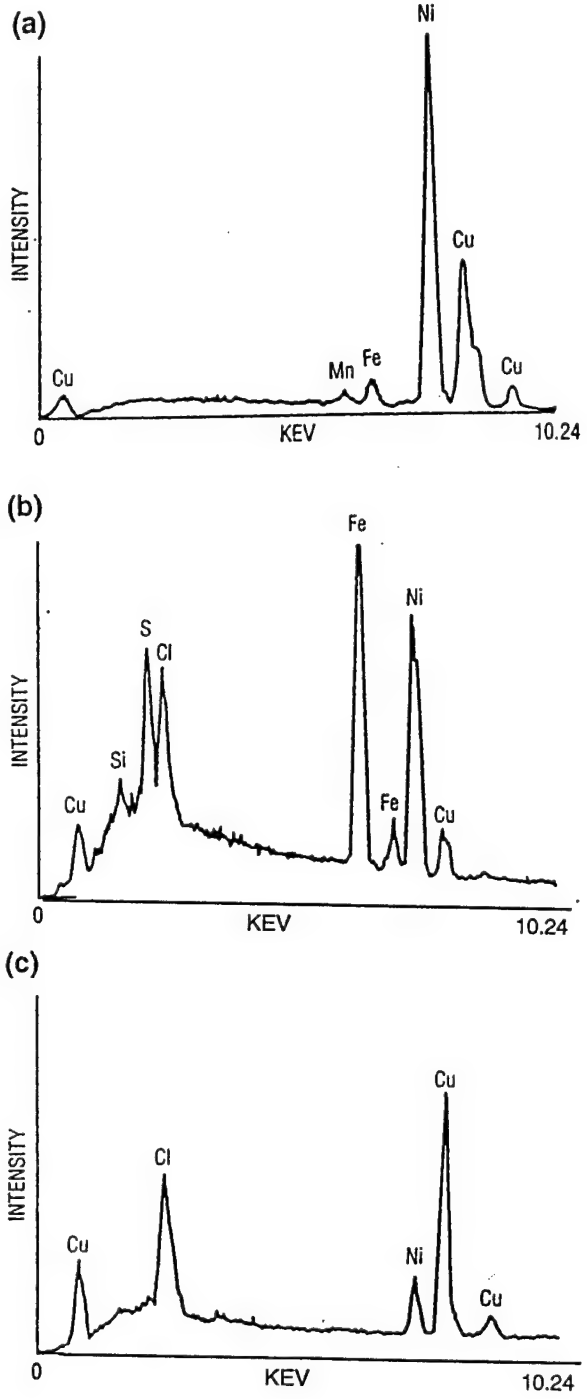


FIGURE 28 - EDS spectra of type 400 nickel alloy: a) unexposed; (b) after exposure to estuarine water for six months showing accumulations of silicon, sulfur, chlorine and iron; and (c) residual metal in the base of pit showing relative nickel depletion and copper enrichment.

Aluminum and Aluminum Alloys

The corrosion resistance of aluminum and its alloys is due to an air-formed aluminum oxide passive film of 0.002 μm to 0.01 μm (20 to 100 Å) thickness. Anodizing produces thicker insulating films that possess better corrosion resistance. The natural film on aluminum can be attacked locally by halide ions, such as chloride. Susceptibility of aluminum and its alloys to localized corrosion makes it particularly vulnerable to MIC.

Most case histories of MIC of aluminum alloys are for aluminum alloy fuel tanks used in aircraft or underground storage tanks. MIC occurs in the water phase of fuel-water mixtures and is most common on the bottom of tanks and at the fuel-water interface. The filamentous fungus, *Cladosporium resinae*, is commonly known as "kerosene fungus" because of its prevalence in water-contaminated fuels.

Contaminants in fuel, such as surfactants, water, water-soluble salts and solids, are considered to contribute to observed corrosion damage. Fuel and its additives may be used as a source of nutrients by the bacteria. Microorganisms in fuel cause filter plugging and fuel degradation. Protective paint coatings used in fuel tanks can be degraded by microorganisms, forming fissures that can cause perforation or that can propagate to cause corrosion fatigue.

Titanium and Titanium Alloys

Titanium and its alloys are corrosion resistant in many environments, including soils and seawater, where it has shown marked resistance to pitting and crevice corrosion. Pitting has only been observed at high temperatures and chloride concentrations. Titanium and its alloys have shown extremely good reliability and absence of corrosion attack in normal heat exchanger tube applications. Considering these outstanding properties and the mechanisms of MIC described in this review, it is not surprising that researchers have found no evidence of MIC in titanium.

MIC Sample Identification and Collection

Examination of a Corroding Metal

MIC testing goals include: determining whether or not MIC has taken place; monitoring a particular situation to determine onset and extent of MIC; or determining the likelihood MIC will take place in particular metal/microbe combinations. Testing differs with the goal. In the following chapters, metallurgical, microbiological/microscopic, chemical, and electrochemical tests will be described that can be used in combination for failure analysis, monitoring, and prediction. In the final chapter, specific recommendations will be made for application of MIC test procedures. When working with chemicals, the user must first consult the material safety data (MSD) sheet and handle the materials accordingly. Safety requirements are available in standard reference sources and will not be specified in this text.

1. Take color photos of corroded areas and identify their relationship to the overall system.
2. Note surface deposits in corroded areas. Describe form, color, texture, and odor.
3. Describe corrosion: pit shapes and sizes, crevices, etc.
4. Describe electrolyte: saline or freshwater, suspended solids, flow rate, direction of low, color, texture.
5. Note environmental factors in corroded areas and upstream: e.g., atmospheric, ground or water pollution, industrial operating cycles.

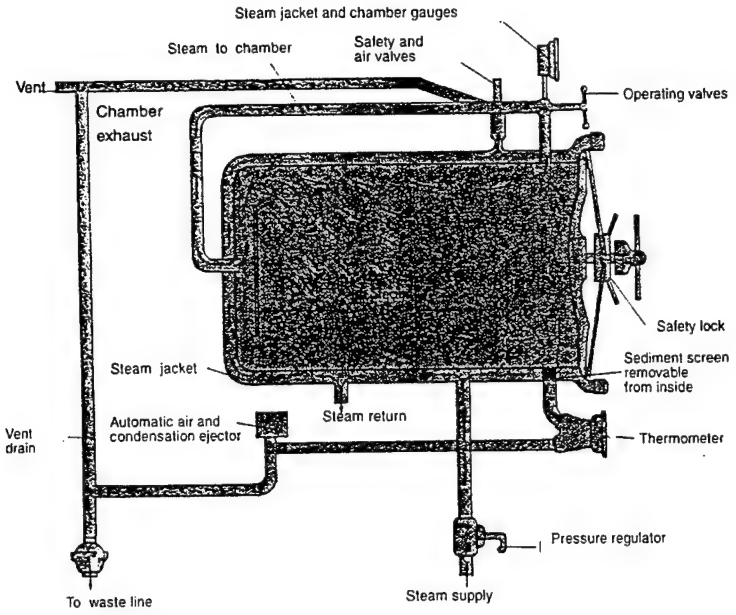


FIGURE 29 - Schematic of laboratory autoclave.[6]

6. Compare overall description with photographs and descriptions of known cases of MIC included throughout this text and *A Practical Manual on Microbiologically Influenced Corrosion.*[3]

Sampling

Collect corrosion products and electrolyte close to corrosion sites using clean, sterile, sealable, carefully labeled containers. Label sample containers with sample origin, date, sampling time, and tests to be performed on the sample. Sterile containers should not contain chemicals that will inhibit microbial survival. Most sampling containers are glass or plastic. In general, glass containers have screw-down caps and are sterilized using a combination of pressure and temperature over time (15 psi steam ~121°C [250°F] for a minimum of 20 minutes). Commercially available devices, called autoclaves, can be manually or automatically controlled to produce temperatures and pressures required for sterilization of liquids and solids (Figure 29).

Pressure cookers also can be used with extreme care. Glass containers can be reused many times if they are cleaned and sterilized before each use. Plastic containers usually are disposable; however, some can be recleaned and autoclaved. In most cases, plastic sampling containers have rigid walls, but polyethylene flexible-walled containers with closure ties can be used. Sterile plastic bags provide lightweight sample containers. Fill to no more than 60% maximum volume. The bag should be sealed tightly and the whole bagged sample sealed again within a second plastic bag to reduce the risk of leakage. Plastic bags designed for domestic use are not sterile and should be used only as a last resort for storage of samples that will not be used for microbiological information. It is essential that extraneous dust, dirt, and debris not be introduced into the sample at the time of collection. Do not directly handle the interior surfaces of sample containers or any other part likely to be in direct contact with the sample.

Sampling should include both sections of the corroded material and the electrolyte to which it was exposed. To collect corrosion products, scrape area with a sterile scalpel or swab the area with a sterile cotton swab. Individually wrapped sterile implements can be purchased from most pharmacies and all scientific supply stores.

Deposits from the corroded areas are the first choice for testing purposes because causative organisms are expected to be found in relatively high numbers at the corrosion site. If possible, remove a section of the corroded area with a hand saw, cutting as far away from the corroded area as practical. Solid samples should be collected with the electrolyte to which they were exposed to avoid desiccation. Scrapings and liquids will be used for the enumeration of microorganisms.

The relationship of microorganisms to corrosion products can be examined with scanning electron microscopy (SEM). Sections for SEM examination should not be disturbed by probing or swabbing. Instead, carefully remove those sections and place in either cacodylate-buffered 2-4% glutaraldehyde or 2-4% glutaraldehyde in filtered (0.2 µm pore size) seawater, depending on whether the specimen has been exposed to freshwater or marine waters, respectively, and chill. Sodium cacodylate is an organic arsenical salt with buffering properties. A 0.1 M solution (21.4 g L^{-1}) is adjusted to pH 7.2-7.4 by the addition of drops of concentrated hydrochloric acid (HCl). The pH range is optimum for the fixative and can be used for samples originating from any pH. Glutaraldehyde is a fixative for histochemical purposes and can be used to fix microorganisms on surfaces for later investigation with SEM. Sodium cacodylate and glutaraldehyde are available from chemical supply companies and should be handled carefully according to directions specified on the MSD sheet.

The second choice for test samples is water or process fluid. Such samples have one major drawback: Numbers and types of organisms present in the fluid (planktonic) may have little correlation to those on surfaces (sessile). If stressful changes are occurring in the system (temperature deviations from the optimum range for growth, diminishing nutrients, etc.), growth on surfaces is favored, and the relative number of planktonic organisms will decrease.

Liquid samples can be collected without introducing microorganisms using a sterile needle and syringe. One limitation to this technique is that the sample is pulled into a vacuum and a temporary shortage of oxygen may be stressful for strictly aerobic organisms.

1. Clean surface of the container into which the sample is to be injected with a clean paper towel moistened with rubbing alcohol or 30% hydrogen peroxide solution. Allow to dry.

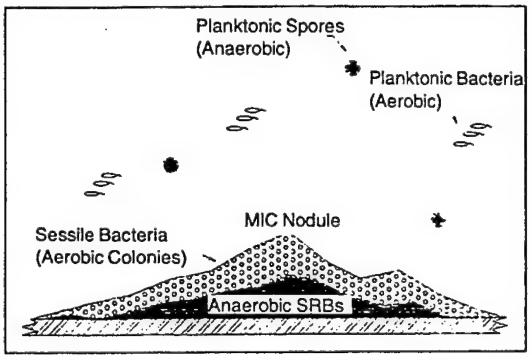
2. Remove cap from the disposable needle and screw the needle onto the syringe. Carefully insert needle into sample liquid and withdraw the syringe plunger slowly. Liquid will be drawn into the sterile syringe.

To collect a sample from a stream of flowing liquid, the container cap should be carefully unscrewed and placed (inside down) on a clean surface. The clean surface is important since dust and dirt on the surface may contaminate the inside of the cap.

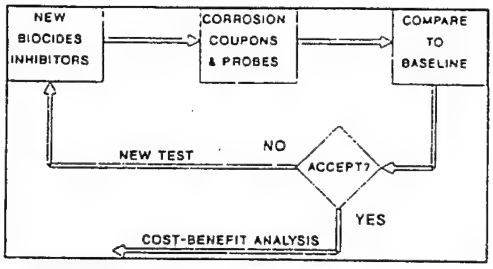
If there are doubts about area cleanliness, surfaces should be swabbed with a 10% dilution of domestic bleach (which, undiluted, contains 5-6% sodium hypochlorite). Allow the surface to dry before beginning the sampling procedure. At time of uncapping, the sampling bottle will have a sterile interior that will be exposed to air. Do not touch inside surfaces; handle container by gripping the lower part of the bottle and avoid any skin contact with the upper part of the container. When taking a water sample, gradually place open container vertically into the water flow and allow the water stream to fill the bottle. Once the bottle is two-thirds to three-quarters filled, move the bottle horizontally out of the water and quickly cap and seal.

Biocides are more effective in killing planktonic organisms than sessile organisms; therefore, samples of systems treated with biocides may indicate low numbers in water samples while there are high numbers growing on surfaces. Water sample testing is a compromise, but sometimes it is the only option, and is preferable to not monitoring a system at all.

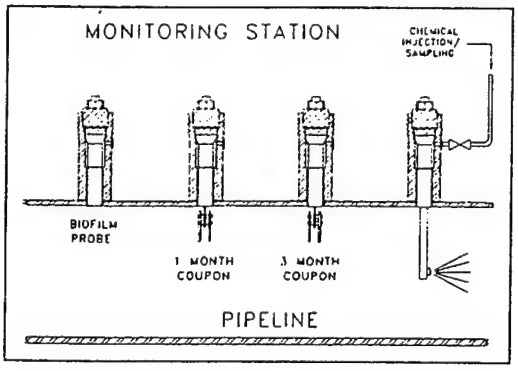
A third sampling strategy involves testing samples of biofilms formed on sampling devices designed to monitor biofouling by periodic removal of sample plugs (Figure 30). Chapter 7 discusses additional monitors.

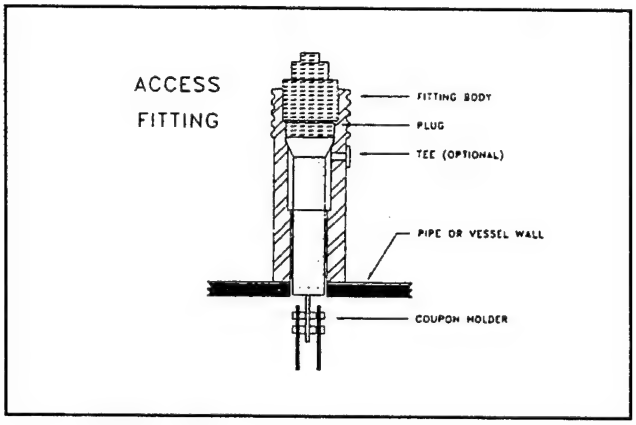


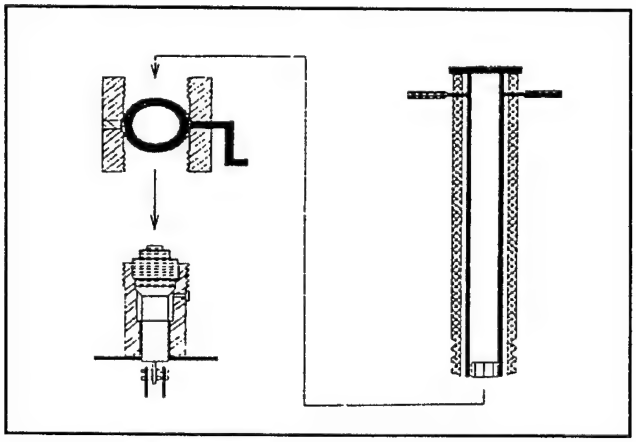
PIPELINE BACTERIAL ACTIVITY



EVALUATION FEEDBACK







ACCESS FITTING, VALVE, AND RETRIEVAL TOOL

FIGURE 30 - Monitoring strategies.[7] (Reprinted with permission of the Institute of Applied Microbiology.)

Examination of biofilms on coupons or sample plugs is useful in monitoring general biofouling tendencies, but its worth is limited because of the patchy nature of biofilms. Sample plugs should either be preserved for SEM examination as described for solid deposits or swabbed for microbiological characterization as described in the Transport section.

Transport

It is preferable to arrive at the collection site with all fixatives and media for serial dilutions. If that is not possible, it is necessary to transport liquids and solids. Aerobic and anaerobic transport culture tubes complete with media are commercially available. The major concern in transporting samples for microbiological evaluation is to ensure that microorganisms remain alive and active without multiplication. Sample collection may expose microorganisms to abrupt changes in pressure, temperature, and light, causing a redistribution in numbers and types of microorganisms in the original sample. While this is not desirable, it is unavoidable.

Transport techniques are designed to minimize alteration of the microbial population. After collection, samples should be stored in the dark away from temperature extremes. When transit times are less than 6 h, liquid samples should be maintained at the original collection temperature by storing in an insulated container. If the electrolyte has been collected from cold water sources with a median temperature below 8°C (46°F), ice packs should be added to the insulated container. If the sample has been collected from sources above 30°C (86°F), intrinsic heat should maintain the microbial population without significant changes. If transit time is longer than 6 h, sample temperature should be lowered to less than 10°C (50°F) to restrict growth.

A standard method used to control post-sampling shifts in microflora is to provide a cold temperature shock by packing ice bags around sample containers to bring the temperature down quickly to within the range of 1 to 4°C (34 to 39°F) where microbial activity will be reduced to a basic survival metabolic mode. Prolonged storage for periods of longer than 24 h under any condition will cause unacceptable changes in the microflora. Samples shipped or transported in glutaraldehyde can be kept refrigerated for several days before further preparation for SEM. The protocol for SEM preparation will be described elsewhere.

CHAPTER 3

Metallurgical Testing

Metallurgical testing has been used extensively to identify potential MIC.[8] Examine the corrosion area carefully and isolate specific areas of interest. Remove surface deposits using a sterile spatula or other sharp edge into a sterile container, being careful not to disturb base metal. After removing as much deposit as possible without damaging the metal, continue cleaning with a dry, stiff nylon brush, such as a toothbrush. If the surface is still not clean, a wire brush may be used. To limit damage to the metallurgy, brush only in the direction of the length of the pipe. Describe the appearance of cleaned corroded areas. Examine corroded areas using a low power (e.g., 5X to 60X) magnifying lens or microscope. The following metallurgical features can indicate MIC:

1. Cup-shaped, hemispherical pits on the flat metal surface and craters up to 2 to 3 in. across. Sometimes the cup-type pits occur on the inside surface of the craters (Figures 13 [page 9], 31, 32).

2. Striation lines (contour lines) that run parallel to the length of the pipe (Figures 33, 34).

3. Tunnels on the upstream and downstream surfaces of the craters that run parallel to the length and rolling direction of the pipe (Figures 35, 36).

4. Rounded pits under discrete mounds or tubercles on carbon steel (Figures 37, 38).

5. Subsurface cavities along weld seams in stainless steels (Figures 39, 40).

6. Localized discrete mounds or deposits.

These metallurgical features have been established after years of observations and failure analyses. This list, however, is continuously updated.[8]

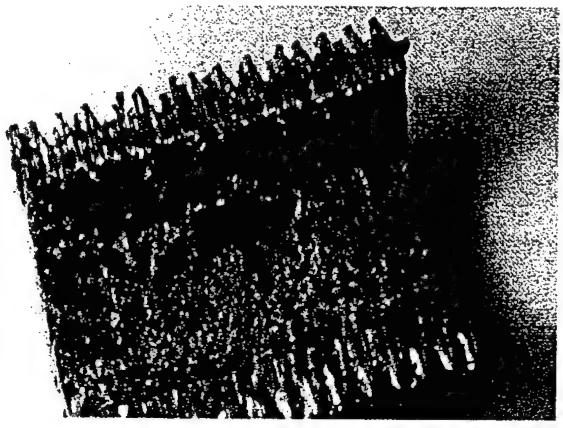


FIGURE 31 - Cup-shaped pits on 90/10 copper-nickel tubing.

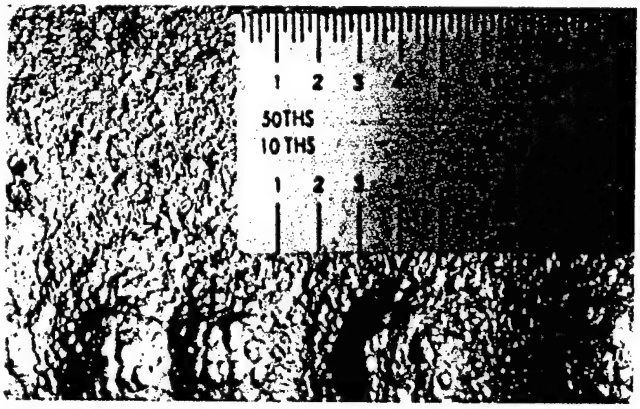


FIGURE 32 - Cup-type hemispherical pits on flat surfaces with craters in pits (carbon steel).[8]
(Reprinted with permission of the Gas Research Institute.)

FIGURE 33 - Pits with striations (carbon steel).[8] (Reprinted with permission of the Gas Research Institute.)

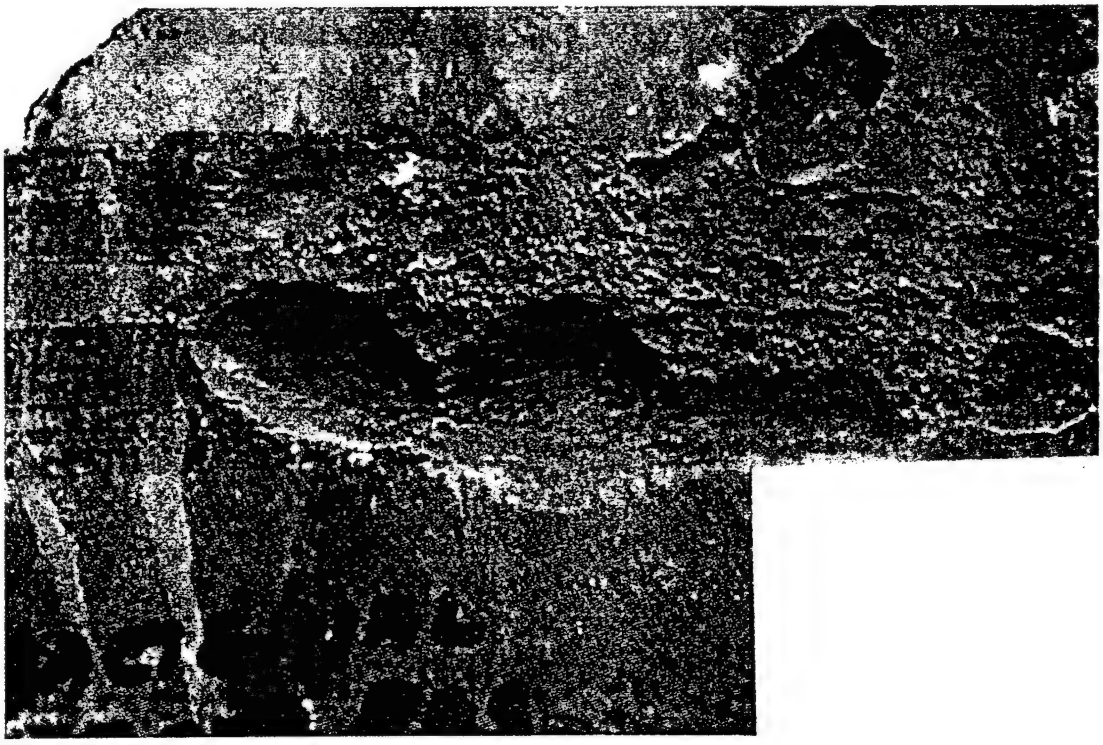


FIGURE 34 - Pits with striations (carbon steel).[8] (Reprinted with permission of the Gas Research Institute.)

FIGURE 35(a) - (right) Cross section of pit in carbon steel showing tunnels parallel to length of pipe (20X magnification).[8]
(Reprinted with permission of the Gas Research Institute.)

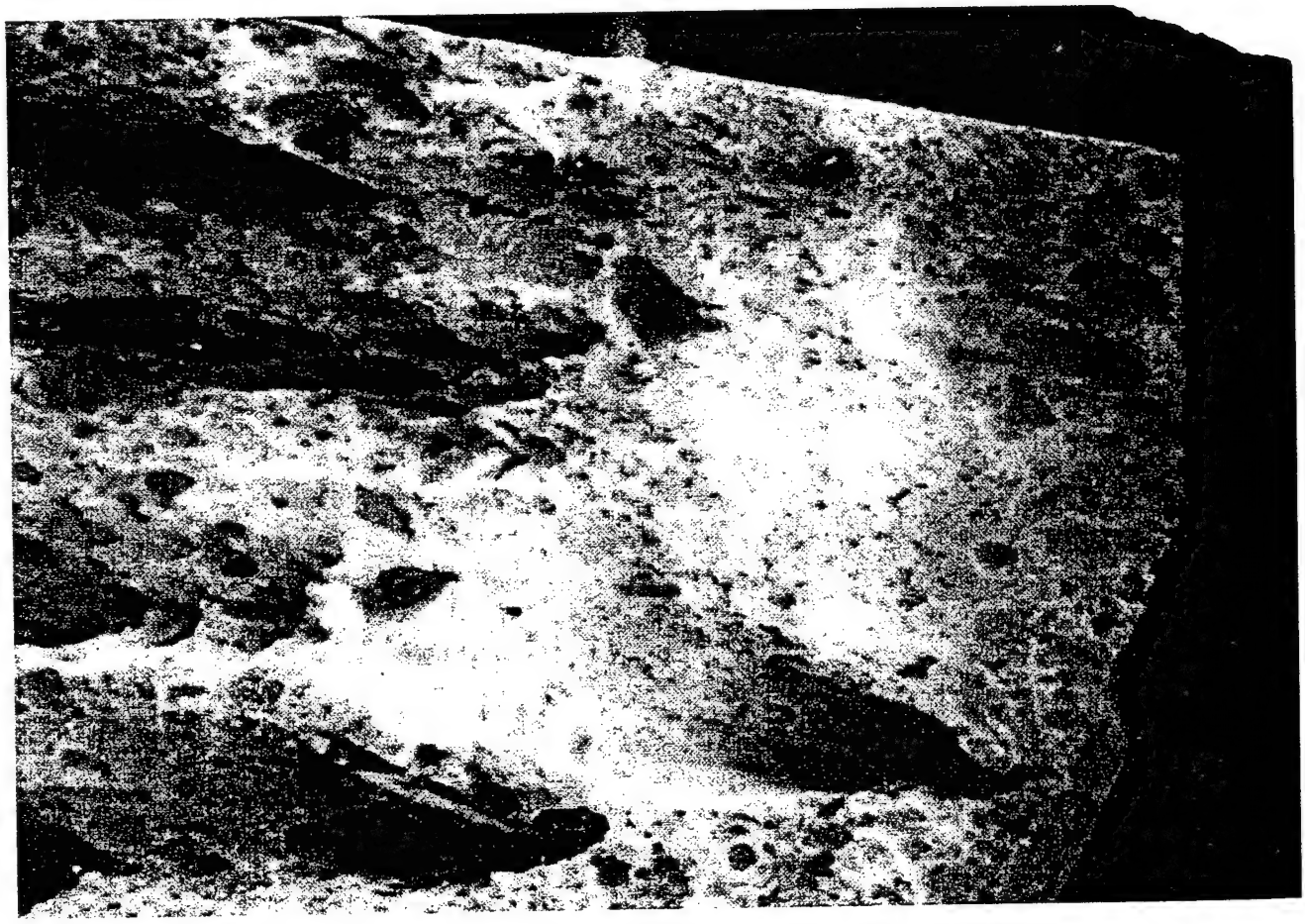
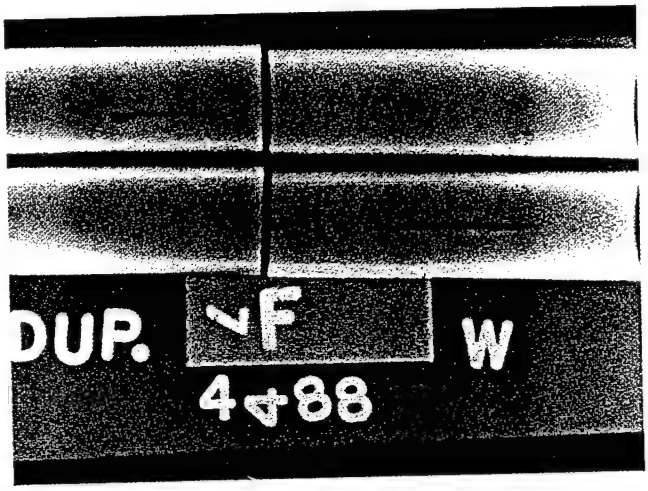
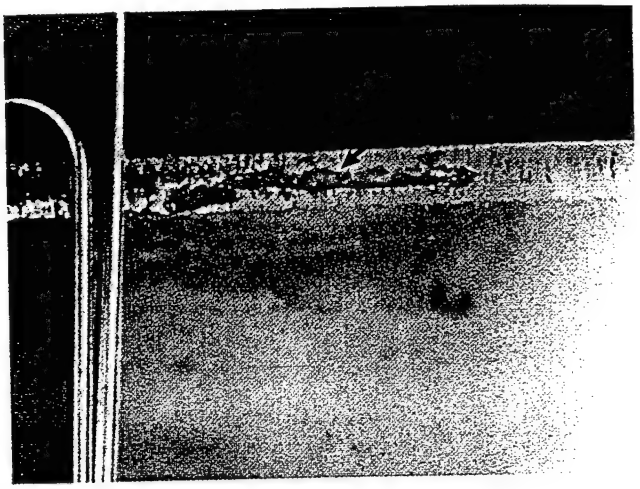


FIGURE 35(b) - 100X magnification of 35(a) showing tunnelings.[8] (Reprinted with permission of the Gas Research Institute.)



36a



36b

FIGURE 36 - (a) (left) Radiograph showing pits on internal surfaces leading to tunnel-like cavities in tube walls in 304 stainless steel, 5/6X and (b) longitudinal cross section through one of the tunnels (arrow) shown in (a). External surface is along top edge of the tube, 3.5X.[3]

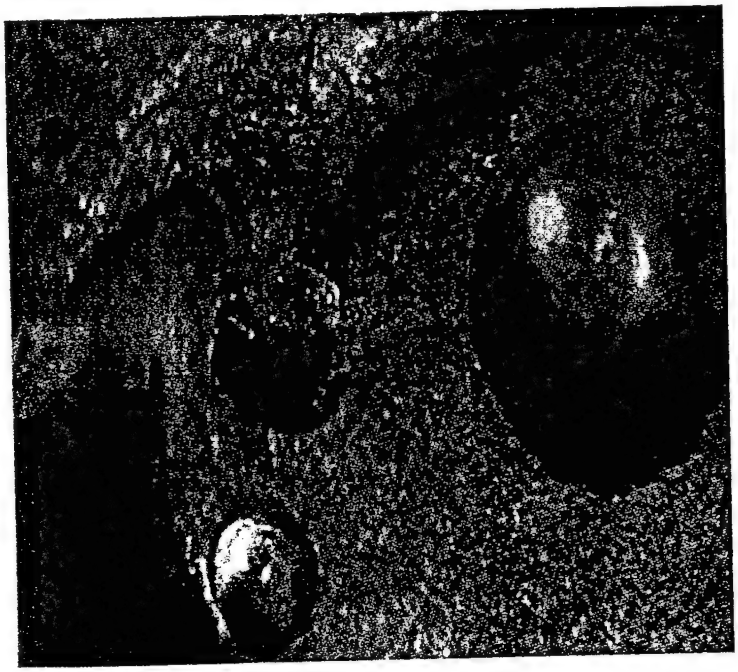


FIGURE 38 - Discrete pits and mounds on carbon steel. 50X.

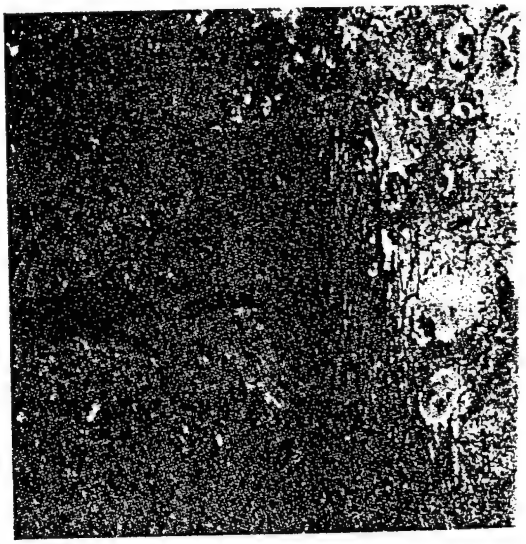


FIGURE 37 - Carbon steel plate from a hot well in a nuclear power plant. Nodules have a rust-colored outer layer with a black interior under which pits are located.[9]

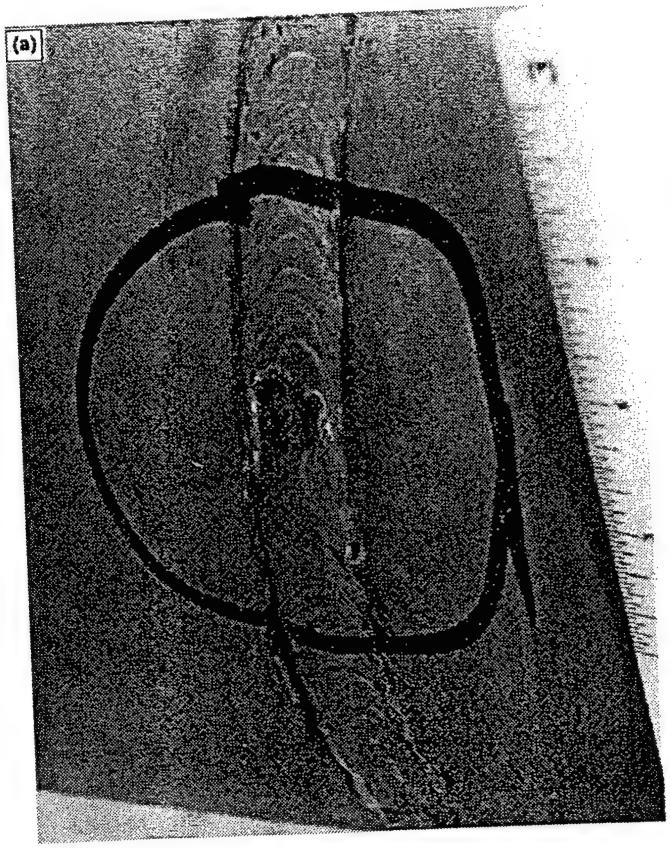


FIGURE 39(a) - Weeper at 308L weld on outside diameter of 316L pipe.[10]

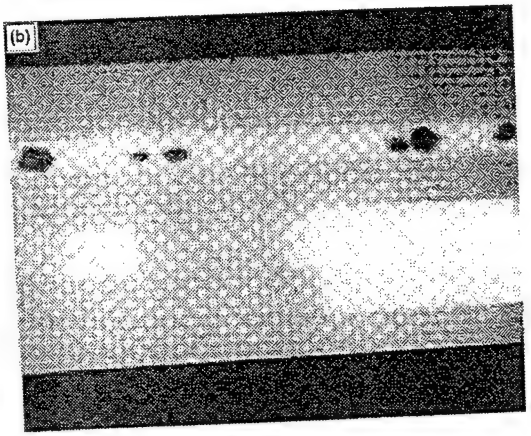


FIGURE 39(b) - Radiograph of the pipe weld.[10]

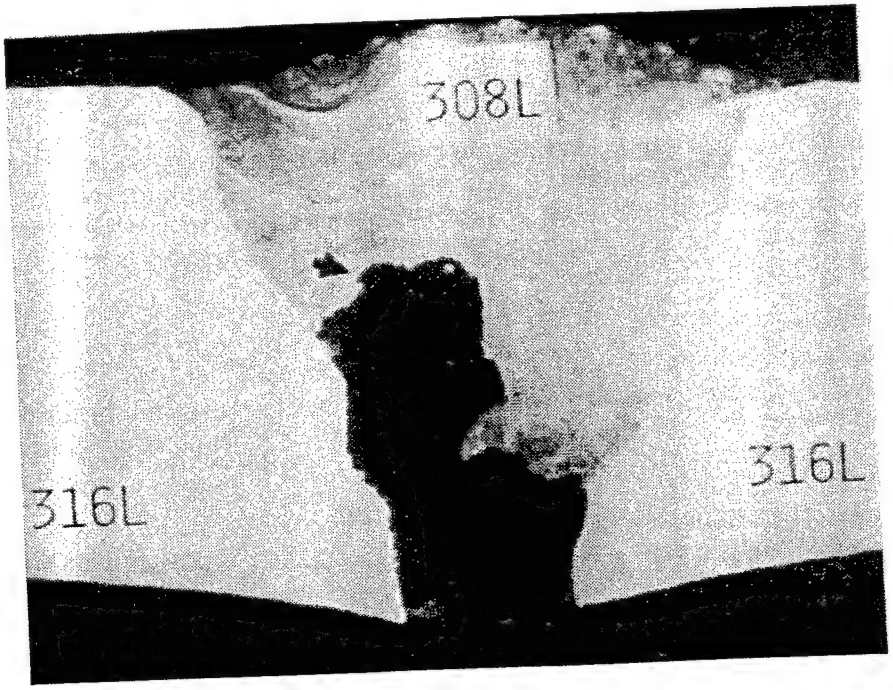


FIGURE 40 - Cross section of pipe weld in Figure 39.[10]

CHAPTER 4

Microbiological Testing

Almost all cases of MIC are accompanied by biologically rich deposits in association with the corroded areas. MIC often is attributed to a single type of microorganism, but more often the corrosion is due to the activities of several different organisms that form a community. One of the essentials in MIC testing is that one establish a spatial relationship between microorganisms and localized corrosion. As previously indicated, numbers and types of microorganisms within biofilms cannot be predicted or determined by measuring planktonic or free-swimming species.

Any method of evaluating the potential for MIC must be capable of quantifying cells in the presence of extracellular polymers, sludge, and corrosion products. Furthermore, while it is recommended that one determine the numbers and types of cells on the surface, a direct correlation between the numbers and types of cells and the likelihood of MIC has never been established. Instead, the number and types of microorganisms associated with corrosion products are used with surface chemistry, electrochemical, or metallurgical data to determine corrosion mechanisms consistent with the observed corrosion.

Even though all types of microorganisms can potentially cause MIC, two specific physiological groups of bacteria have been shown to be most closely related to localized corrosion in aqueous media: SRB and iron-depositing bacteria.

The gram staining technique is one common method used to classify bacteria into two groups, Gram negative and Gram positive. It is based on the ability of some bacteria to take up and retain certain dyes. The Gram stain procedure involves the application of a basic purple dye, gentian or crystal violet, which stains all bacteria able to absorb this dye. Next, a dilute solution of iodine is added, serving to decrease the solubility of the purple dye within the cell by combining with the dye to form a dye-iodine complex. An organic solvent, such as ethanol, which readily removes the purple dye-iodine complex from some, but not other, species of bacteria is added next. A red stain is then applied. Bacteria decolorized by the ethanol appear red and are classified as Gram negative. Those that retain the purple dye will appear purple and are classified as Gram positive. The differing responses are related to the chemical structure of the cell wall and distinctive cell properties.

To grow or culture microorganisms to numbers that can be counted and identified, a small amount of liquid or a suspension of a solid is added to a solution that contains nutrients in accordance with standard microbiological procedures (see any microbiology reference book, e.g., *The Procaryotes*, Springer-Verlag, 1981). The small sample is called an inoculum, and the nutrient solution is called the growth medium. Media can be extremely complex with trace minerals and vitamins, or simple, containing only a carbon source and water.

The distinct advantage of culturing techniques is that they are extremely sensitive. Low numbers of cells grow to easily detectable higher numbers in proper culture media. However, growth media tend to be strain-specific. Incubation at a single temperature is further selective. It must be noted that the goal of culture techniques cannot be to determine the exact number of microorganisms in a specific sample.

No growth medium can approximate the complexity of a natural environment. Instead, it is important that

one can establish the presence and approximate numbers of specific types of organisms and that one use a standard methodology so comparisons can be made.

Documentation of Microorganisms

As part of the microbial community in aquatic systems, bacterial populations are actively involved in nutrient cycling. The significance of these populations often is difficult to ascertain because of the presence of many physiological types. However, measurement of bacterial densities by microscopy or total plate counts usually is the first step in trying to establish any relationship that might exist between microorganisms and corrosion.

All types of high-powered microscopes can be used to detect microorganisms; however, epifluorescence microscopy and SEM have most often been used in MIC studies. The following method describes a procedure for detection and enumeration of aquatic bacteria using acridine-orange epifluorescence direct-microscopic counting (Figure 41). Applications include cooling tower waters, process waters, and waters associated with oil drilling wells. Microorganisms in aqueous media should be preserved to prevent either an increase or decrease in the number of cells due to multiplication or lysis, respectively. Water samples may be preserved with 0.2 mL 10% formaldehyde/10 mL sample.

Certain types of debris and other microorganisms may fluoresce in acridine orange-stained smears. The test method requires some experience to distinguish bacteria from other fluorescing bodies on the basis of morphology when viewed at high magnifications. A minimum of 10 cells mL^{-1} is required for detection by this test method. Aquatic bacteria can be enumerated by filtering a known quantity of water through a 0.2 µm polycarbonate membrane filter. Cells retained on the membrane filter are stained with acridine orange solution and examined for fluorescing bacteria cells using a fluorescent microscope. Acridine orange epifluorescence direct-counting procedures cannot differentiate between viable and nonviable cells.

The following procedure cannot be used to convert cell numbers directly to total carbon biomass due to natural variations in bacterial cell size. Equipment and reagents required for epifluorescence microscopy include:

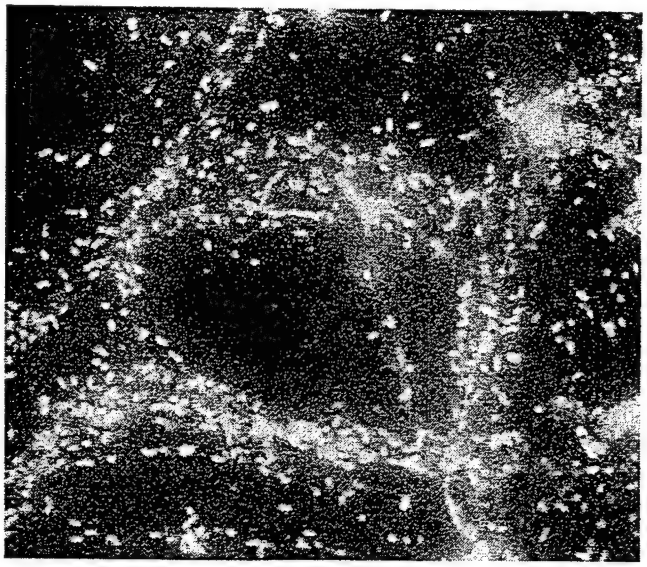


FIGURE 41 - Epifluorescent miscroscopy of corrosion deposits, 400X. (Courtesy R. Prasad, Champion Technologies, Inc.)

- Fluorescence microscope, with oil-immersion objective lens (100X);
- Eyepieces, 12.5X, equipped with a net micrometer;
- Condenser, 1.25X;
- High-pressure mercury lamp, 200 W on a ultraviolet light source giving vertical illumination and a filter unit with BG12 and BG38 transmission filters or equivalent;
- Stage micrometer;
- Membrane filter support (25 mm), sterile, particle-free, fritted-glass;
- Funnel, 15 mL capacity or equivalent;
- Membrane filter, sterile plain regular polycarbonate, 25 mm (0.2 µm pore size);
- Filter apparatus containing vacuum source, filter flask, and a filter flask as a water trap;
- Forceps (flat tip), alcohol, Bunsen burner, clean glass slides, and cover slips;
- Phosphate buffer solution – dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL water. Adjust to pH 7.2 ± 0.05 with

sodium hydroxide (NaOH) solution (40 g L^{-}) and dilute to 1 L with water;

- Acridine orange solution – dissolve 10 mg acridine orange in 100 mL phosphate buffer; filter acridine orange solution through a 0.2 µm filter before use;
- Isopropanol;
- Xylene;
- Immersion oil, very low fluorescing equivalent to Cargille Type A.

Enumeration of cells is accomplished using the following procedures:

- Place a 0.2 µm membrane filter on filter base and attach funnel. Add 10 mL water sample. Turn on vacuum.
- Rinse membrane with 5 mL sterile reagent water (ASTM D1193 Type 1A).
- Turn off vacuum and flood membrane with acridine orange solution. Allow to stand for 3 to 4 minutes then turn on vacuum and filter.
- Rinse membrane with 0.5 mL isopropanol. Do not exceed 10 s contact time.
- Rinse membrane with 0.4 mL xylene.
- Remove membrane and air dry for 15 s.
- Place membrane on a clean microscope slide on which two drops fluorescence-free immersion oil has been added.
- Place a drop of immersion oil on top of membrane and apply cover slip.
- Count cells using incident fluorescent illumination in violet light wavelength range (410 nm). Count 20 fields at random within the stained portion of the membrane. Count only that portion of the field within the micrometer area.
- Calculate average number of bacteria per micrometer area.
- Bacterial densities are calculated for 25 mm filters as follows:

Bacterial density mL^{-1} sample = $(2.37 \times 10^4 n/d)$

where:

n = average number of bacteria per net micrometer field; that is [(total number of bacteria counted)/(number of micrometer fields counted)], and

d = dilution factor.

2.37×10^4 is the membrane conversion factor based on a magnification of 1,562.5 (eyepiece 12.5X) x (objective 100X [condenser unit 1.25X]). Wet area is determined by measuring internal diameter of the funnel.

One of the most frequently used techniques for documenting the presence of microorganisms in association with localized corrosion is SEM. As mentioned in the sample collection section, specimens for SEM evaluation should be preserved as soon as possible and kept refrigerated until processed. Small specimens of metal, scrapings, and filtered samples can be evaluated with SEM. Sample preparation is described in Figure 42 and on the next page.

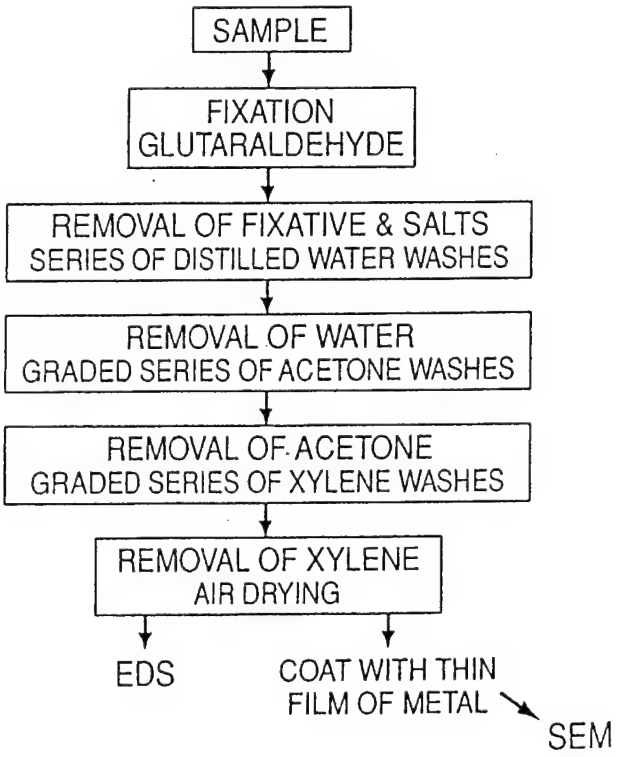


FIGURE 42 - Flow chart for sample preparations for SEM.

- Remove pipe or tube section with a clean cutting possible. Chamber sizes for SEM vary, but final sections should be approximately 2.5 cm^2. Final subsectioning can be completed after dehydration.
- Place samples from freshwater sources in cold 2-4% cacodylate buffered glutaraldehyde for a minimum of 4 h. Samples can remain in fixative for several days. For marine samples, glutaraldehyde can be prepared in seawater filtered through a 0.1 µm filter. Natural seawater contains a carbonate buffer so no additional chemicals are needed.
- Remove water and glutaraldeyde through a series of washes. Beakers containing wash solutions should be placed in an ice bath. Most samples fixed in glutaraldehyde are refrigerated during fixation. Further manipulation should be completed in an ice bath to avoid artifacts introduced by temperature shock. Glutaraldehyde will polymerize at elevated temperatures. Furthermore, removal of fixatives and salts, as well as solvent extractions of water, should be gradual processes, best accomplished at reduced temperatures. Typically, with large environmental samples, the wash solutions include distilled water if the sample was collected from a freshwater source or a graded series of filtered seawater/distilled rinses to distilled water if the sample was collected from a brackish or marine source. It is essential to remove salts from the sample gradually to avoid osmotic disruption. Residual salts will crystallize and obscure cells within biofilms. Distilled water rinses should be followed by a graded series of water/acetone washes (100:0, 75:25, 50:50, 25:75, 0:100 v/v water/acetone) until the sample is in full strength acetone. Removal of acetone is accomplished through a series of graded washes of acetone/xylene (100:0, 75:25, 50:50, 25:75, 0:100 v/v acetone/xylene) to 100% xylene.
- Air-dry specimens in a clean environment.
- Before the specimen can be observed in a traditional SEM, it must be coated with a conducting film of carbon or metal (i.e., gold or palladium) either by vacuum evaporation or sputter-coating.

There are many techniques for fixation, dehydration, and coating specimens for SEM. The method cited above is adequate and requires the least amount of equipment. Operation of any SEM requires an experienced operator; operating conditions will not be defined here. Furthermore, recognizing bacteria within corrosion products requires experience. Figures 43-46 are micrographs of bacteria associated with corrosion. Despite the large body of MIC data derived from SEM micrographs, sample preparation does introduce artifacts and removes material from the surface. The development of the environmental scanning electron microscope (ESEM) means that wet, nonconducting samples can be imaged. The ESEM is not a routine analytical instrument and will not be discussed at any length in this section. (For a brief description of the ESEM, see Chapter 8).

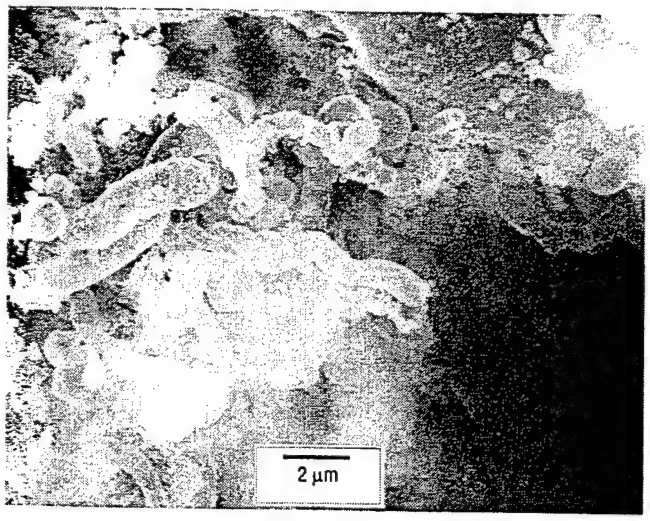


FIGURE 43 - Bacteria from mixed facultative biofilm on copper foil.

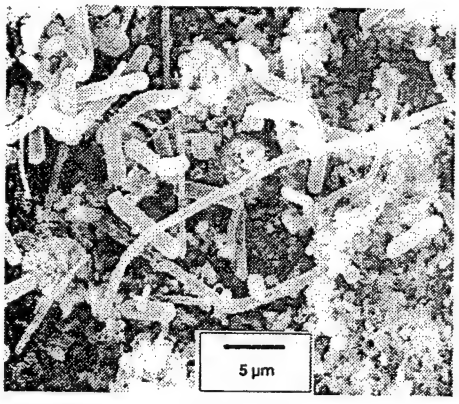


FIGURE 44 - Bacterial cells within biofilm, including filamentous forms, on brass foil.

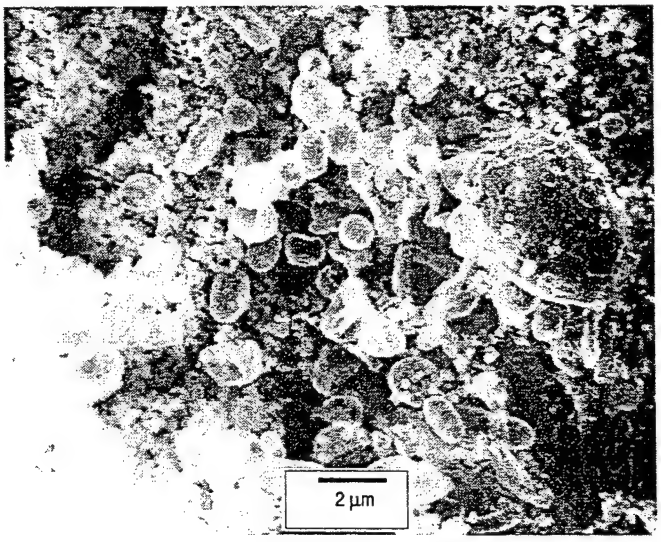


FIGURE 45 - Bacterial cells from mixed culture on copper.

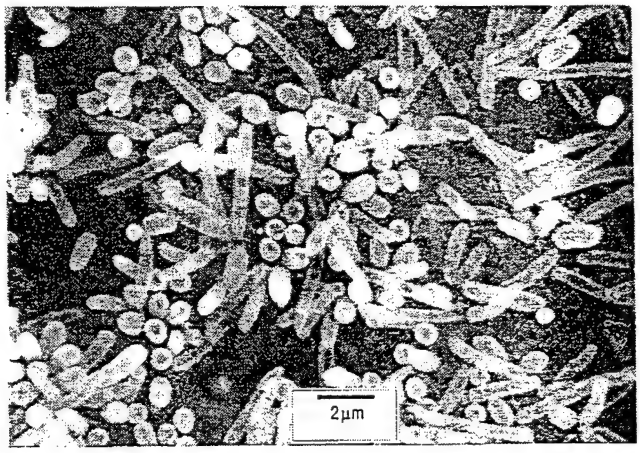


FIGURE 46 - Bacterial cells within biofilm on copper.

It often is necessary to report the size of the viable bacterial population in a sample. A viable count assumes that a visible colony will develop from each organism. The most common technique for counting viable microorganisms is the extinction dilution/spreadplate enumeration using solid agar in plastic, sterile 100 mm diameter Petri dishes. In conducting a spreadplate enumeration from a liquid sample, these steps are required: (a) selection of an appropriate agar culture medium, (b) vortexing liquid to disperse particles, (c) serial dilution of the liquid, (d) dispensing diluents onto agar surfaces, (e) incubation under the correct temperature and sphere (reductive or oxidative), (f) counting visible colonies, and (g) computing the colony-forming units (cfu) per volume or area. These steps are described below.

Enumeration techniques were developed in the medical and food industries to determine numbers of pathogenic and putrefying microorganisms that grow in environments generally very rich in nutrients. Consequently, traditional agar culture media are high in nutrients. Commonly, the major sources of organic nutrients are added in the concentration range of 0.1 to 5.0%. In unpolluted natural waters, it is rare to find environmental conditions in which the organics are in concentrations in excess of 0.001%. Exposure of microorganisms from nutrient-poor environments to nutrient-rich culture media can be traumatic or toxic. Such organisms are organo-sensitive (i.e., cannot tolerate radically high concentrations of organics). Culture media should be selected to approximate the original environmental organic conditions.

Agar is a galactoside derived from marine algae. Most microorganisms cannot degrade agar. Agar is typically used at concentrations between 1.0 and 2.0% (w/v) depending on the formulation of the medium. Agar remains a solid at temperatures up to approximately 90°C (194°F), allowing incubation at high temperatures. Agar solidifies from the molten state at 46°C (115°F).

Each agar culture medium is developed to enumerate a particular spectrum of the microflora, and no single medium is capable of supporting growth of all microorganisms. As previously discussed, there are broad spectrum and selective forms of agar media that serve very different purposes. Most microbial activity commonly occurs within a pH range from 6.5 to 8.75 while most culture media are adjusted to a range from pH 7.2 to 7.4. For media selections other than those recommended for SRB and iron-depositing bacteria and those listed below, consult a *Difco Manual*[11] or *Bergey's Manual.*[12] In subsequent sections, specific formulae will be provided for media preparation. In all cases, reagent-grade chemicals should be mixed with distilled or demineralized water.

Broad spectrum culture media generate a set of nutritional conditions in which a wide variety of microorganisms may be able to flourish (e.g., the heterotrophic bacteria). These are commercially available broad spectra media:

- Brain heart infusion agar/4 is quarter-strength brain

heart infusion agar. Bacterial colonies tend to grow rapidly and to a large size. It is a useful medium to obtain a generalized enumeration of aerobic bacteria. If placed under anaerobic conditions (e.g., through the use of an anaerobic jar and gas pack), colony counts for anaerobic bacteria can be obtained.

- Peptone glucose yeast agar is a major medium used to enumerate heterotrophic bacteria.

- R2A agar is a complex medium that can support a range of aquatic heterotrophic bacteria.

- Czapek-Dox agar can be used to culture most fungi, particularly *Aspergillus* and *Penicillium*. Fungal colonies appear to have a rough, almost cotton-like appearance spreading over the agar surface.

- Potato Dextrose agar allows enumeration of fungi, including both yeast and molds. Yeast form large (3 to 6 mm diameter) colonies with domed profiles and may be pigmented a pink color. Some yeast emit fruity odors (esters) that are very distinctive. A broad range of fungi will grow well on this medium.

Selective culture media use inhibitory chemicals (e.g., antibiotics) and restrictive nutritional regimes to encourage and support growth of a narrow spectrum of microorganisms. Indicators, such as dyes, also may be included to differentiate among surviving species.

An example of a selective culture medium is manganese agar that incorporates both ferrous and manganous forms of iron and manganese along with citrate to encourage growth of metal-depositing bacteria.

Microorganisms in environmental samples usually are enmeshed in particulates. Disperse particles so that colony-forming units are separated to maximize the accuracy of spreadplate enumeration. Water samples should be mixed by shaking or stirring for 10 to 60 s just prior to dilution.

The objective of serial dilutions is to dilute samples in a sterile solution (commonly Ringer's solution) to ensure sufficient cfu to produce a statistically satisfactory number of counts. Ringer's solution is an isotonic solution of three chlorides composed of 8.6 g sodium chloride, 0.30 g potassium chloride, and 0.33 g calcium chloride in 1,000 mL water. Dilutions are made in a tenfold series to and beyond extinction. Extinction occurs when no colonies form on the spreadplate because the dilution is so great that no viable entities are in the volume streaked on the final spreadplate. Dilutions are referred to in the tenfold dilution sequence by the symbol 10^{-x}, where x refers to the incremental dilution. For example, 10^{-3} refers to the third dilution or 1:1,000 dilution. These specific directions are excerpted from Collins and Lyne.[6]

- Pipet 9 mL volumes of sterile diluent solution into sterile test tubes with suitable caps to prepare dilution blanks. Do not fill tubes and then sterilize unless screw-capped bottles are used. Autoclaving tubes with aluminum or polypropylene caps may alter the volume.

- Mix sample by shaking. Use a straight-sided pipet and a pipetting bulb to remove 1 mL. Deliver into the first dilution blank. Discard pipet. With a fresh pipet, dip half an inch into the liquid, draw sample into the pipette, and flush 10 times to mix, but do not blow bubbles. Raise and drain pipet. Remove 1 mL and transfer to the next dilution blank. Discard pipets. Continue for required number of dilutions, and remember to discard each pipet after delivering its contents, otherwise residual liquid on previously used pipets will contribute to cumulative error. Dilutions will be:

Tube No.	*1*	*2*	*3*	*4*	*5*
Dilution	1:10	1:100	1:1,000	1:10,000	1:100,000
Vol. of original sample / mL	0.1	0.01	0.001	0.0001	0.00001
or	(10^{-1})	(10^{-2})	(10^{-3})	(10^{-4})	(10^{-5})

Dilutions are added to agar surfaces by pipet, dropper, or syringe. Three volumes are routinely used for spreadplates: 1.0, 0.5, and 0.1 mL. The latter two volumes require a correction (X2 and X10, respectively) to the standard formula for computing population. Three methods are described for plating material. The accuracy of each depends on separating viable units of bacteria so each will grow into a separate colony.

- The pour plate method requires that the dilution be added to liquid agar. Melt several tubes containing 15 mL agar. Place in water bath at 45-50°C (113 to 122°F) to cool. To melted medium at 45°C, add volumes of

the dilution, mix by rotating tube between palms of hands, and pour into a Petri dish. Make replicate pour plates of each dilution for incubation at suitable temperatures. Allow medium to set, invert plate, and incubate. This technique is not widely used for environmental samples because the heat shock caused by adding organisms to cooling molten agar can be traumatic.

- The spreader method requires solid media in Petri dishes. Place dilution droplet in the center of a plate and spread it over the agar surface by pushing a glass spreader backward and forward while rotating the plate. Replace Petri dish lid and leave for 1-2 h to dry before inverting and incubating.

- In the streaking method, a sterile inoculating loop is used to deliver one loopful of the dilution sample on the solid agar surface and spread it back and forth in a pattern to achieve further dilution (Figure 47).

Individually wrapped, disposable sterile plastic inoculating loops can be purchased from scientific supply stores. Disposable loops can only be used one time. Platinum loops can be used repeatedly but must be sterilized prior to each use. To sterilize the platinum inoculating loop, pass it through a flame until it glows. Flame the loop and spread from area 1 over area 2 with parallel streaks. Flame the loop again and repeat with area 3, and so on. Invert plates and incubate. When streaking methods are used, two loops are useful; one is cooling while the other is being used.

To a large extent, the type of medium used to culture microorganisms determines the numbers and types of microorganisms that will grow. Another major consideration is the temperature under which the inoculated plates are incubated. Temperature can affect the range and rate at which microflora adapt and grow. The incubation temperature should be within the same general range as the original habitat.

Microorganisms are sensitive to the reduction/oxidation (redox) potential of the environment in which they are growing. In the typical aerobic plate count, the surface of the agar is saturated with atmospheric oxygen, encouraging aerobic growth and excluding growth of strictly anaerobic organisms. The atmosphere around the spreadplate can be modified prior to incubation to remove oxygen from the incubator. On occasions, the replacement gas may be a combination of carbon dioxide, methane, nitrogen, and hydrogen in various ratios to allow anaerobic, oxygen-sensitive microorganisms to grow.

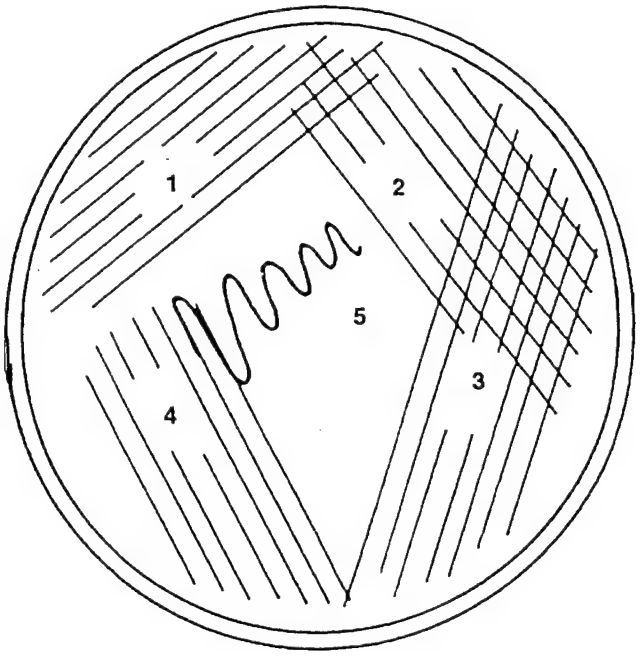


FIGURE 47 - Streaking plate.[5]

An anaerobic container can be prepared using a large-mouth jar into which plates are placed with a burning candle. Secure the lid. The flame will consume available oxygen and extinguish, leaving an anaerobic environment. Incubation times can vary over a wide range, but should last a minimum of 14 days. There is a conflict between the need to get data as quickly as possible and allowing enough time for microflora to adapt and grow.

Enumeration of all visible colonies is required for a total plate count. To count on a simple colony counter, select plates with 30 to 300 colonies. Place open dish upside down over the illuminated screen. Count colonies using a 75 mm magnifier and a hand-held counter. Mark the dish above each colony with a felt tip pen. Calculate the colony or viable count mL^{-1} by multiplying the average number of colonies per countable plate by the reciprocal of the dilution. Report as cfu mL^{-1}.

For large workloads, semi- or fully automatic counters are essential. In the former, the pen used to mark the glass above the colonies is connected to an electronic counter that displays numbers counted on a small screen. In fully automatic models a TV camera or laser beam scans the plate and results are displayed or recorded.

Specific Microbiological Testing

In addition to determining the total viable bacterial population as previously described, it often is desirable to determine specific types of bacteria within the total population. In the following sections, specific tests will be described for bacteria that have been determined to influence corrosion processes.

1. Sulfate reducing bacteria (SRB)

SRB can have several morphologies and diverse nutritional requirements. They have in common the capacity to reduce sulfate to hydrogen sulfide. Hydrogen sulfide can react with metals to produce metal sulfides as corrosion products. Typically, metal sulfides are black. *Desulfovibrio*, one genus of SRB, can form as much as 10 g of sulfide L^{-1} during active multiplication.

Standard practices for evaluating the contribution of SRB to corrosion processes depend on the detection and quantification of SRB using culturing techniques that enumerate organisms or quantify intrinsic characteristics of SRB, including enzymes and antibodies. In addition, one should note the presence of hydrogen sulfide odor (i.e., rotten egg smell) and blackening of slime layers (Figure 24, page 14), metal, electrolyte, or corrosion products to verify presence and activity of SRB.

The object of the following test procedures is to quantify SRB; yet there is no specific number that would be indicative for SRB-influenced MIC. Seawater typically contains 10^0 to 10^1 mL^{-1} SRB irrespective of depth, with 10^2 to 10^5 mL^{-1} typical in bottom muds. Concentrations of SRB as high as 10^9 to 10^{10} mL^{-1} are typical in anaerobic corrosion products. As in all cases of microbiological testing, increases in numbers of cells are important.

A common method for evaluation of the number of organisms in a sample is the Most Probable Number (MPN) procedure. MPN estimates are based on the asumption that microrganisms are randomly distributed in liquid media; that is, repeated samples of the same size from one source are expected to contain the same number of organisms on average.

Assuming that any viable organisms present in a specific sample will grow in the medium used and that distribution within the sample is random, statistical probability theory can be employed to calculate the MPN. A general equation has been derived and standard tables of MPN calculated results have been prepared based on specific combinations of inoculum size, diluent volume, and number of tubes inoculated, giving the MPN of organisms per 100 mL sample for various combinations of observed positive tubes.

Precision of MPN estimates increases as a larger number of tubes are used for each dilution. If the approximate microbial population is not known, a minimum of five tubes of at least a 1:10 dilution is recommended. It is important to note that tables in the literature apply only to specific numbers of tubes and indicated dilutions; consequently, it would be advisable to consult a reference before planning dilutions. For description of techniques and the tables for determining MPN values, see references 6 and 14.

Culture techniques for SRB require an anaerobic environment and an appropriate medium. Anaerobic jars made of metal or plastic use "cold" catalysts, (palladium pellets, Figure 48) that should be kept dry, dried after use, and replaced frequently.

For ordinary clinical laboratory work, commercial sachets are a convenient source of hydrogen and hydrogen-carbon dioxide mixtures. Compressed hydrogen gas also can be used. The cylinder must be fitted with a suitable reducing valve and pressure gauge. Mixtures of hydrogen and carbon dioxide or these gases mixed with nitrogen are safer. Commercial anaerobic cabinets are available for work with anaerobes. Culture medium is pre-reduced, inoculated, and incubated in an inert, oxygen-free atmosphere.

Several proprietary liquid SRB media can be purchased (MICKIT III-C™, Bioindustrial Technologies, Georgetown, TX; and Petrolite Co., St. Louis, MO). The important thing for the individual investigator is to use an SRB culture medium consistently. The most convenient method is to purchase prepared, premeasured, presterilized media. One can make media by buying the necessary reagents, preparing aliquots, and sterilizing. There are innumerable formulae for SRB media. Several were published by Postgate.[13]

The following describes preparation of one lactate-based SRB growth medium that will sustain growth of lactate-oxidizing SRB, but not acetate-oxidizing SRB. Attempts to prepare all-purpose SRB media have been unsuccessful. There is no correct SRB medium. Instead, selection is based on personal preference, availability, and results.

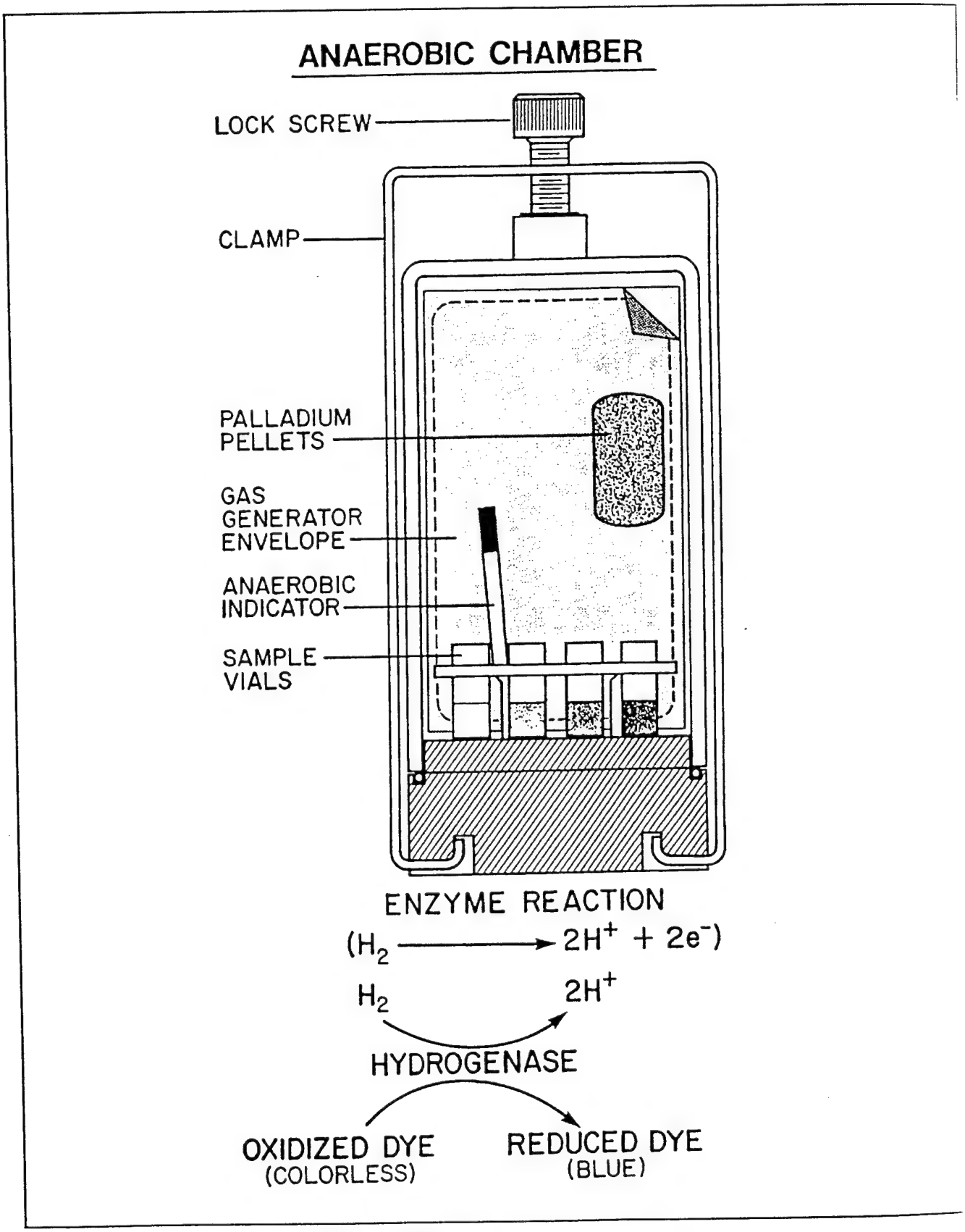


FIGURE 48 - Commercial anaerobic chamber.

Postgate's B medium

Solution 1:

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$CaSO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
sodium lactate	3.5 mL
yeast extract	1.0 g
NaCl	25.0 g (for marine bacteria only)

Add above ingredients to 960 mL distilled H_2O; pH will be 7.2-7.8

Solution 2:

ascorbic acid	0.1 g
thioglycolic acid	0.3 g

Add above ingredients to 20 mL distilled H_2O, adjust pH 7.4-7.8 with 4.0 N NaOH.

Solution 3:

$FeSO_4 \cdot 7H_2O$	0.5 g

Add to 20 mL distilled H_2O. Acidify to pH 1.8-2.0 using HCl, adding dropwise until it dissolves.

Autoclave the three solutions separately. After about 5 minutes cooling, combine the three solutions (if going to use immediately). Let cool. Solutions may be stored separately under refrigeration and heated and combined when used.

Dilution procedures:

Enumeration of microorganisms is based on a specific reaction in a serial dilution. SRB media are typically dispensed in 9.0 mL aliquots into bottles or tubes plugged with rubber stoppers. Use these procedures:

a) Label a series of bottles or tubes with numbers 1, 2, 3, 4, 5, 6, etc. It is impossible to know how many dilutions are required, but the goal is to select a number so that the final dilution will not have a positive reaction. In that way, one can be certain not to underestimate the population. Add 9.0 mL sterile SRB medium to each. Swab rubber stoppers with alcohol.

b) Draw liquid test sample into a sterile container.

c) Remove a sterile syringe from its plastic container.

d) Immerse needle tip in the liquid sample and fill syringe.

e) Point tip upward and force out all but 1 mL of liquid, being sure that no air is trapped in the syringe.

f) Invert first bottle, insert needle through the rubber stopper, and dispense liquid.

g) Without removing the syringe, shake bottle to mix the SRB medium with the liquid sample.

h) Withdraw 1.0 mL of the mixture from the first bottle into the syringe and inject it into second bottle.

i) Shake and draw 1.0 mL of the mixture from the second bottle into the syringe and inject it into third bottle.

j) Continue until last bottle has received 1 mL from previous bottle.

k) Keep bottles at room temperature and observe daily up to 28 days.

l) Positive results are indicated by production of visible black iron sulfide formed from the reduction of $FeSO_4$.

m) Interpretation: The number of bottles showing positive results within the allotted time period can be used to calculate the number of SRB in the original sample by means of the following:

No. Positive Bottles	Bacteria mL^{-1} Original Sample
0	0
1	1–10
2	10–100
3	100–1,000
4	1,000–10,000
5	10,000–100,000
6	100,000–1,000,000

Solid corrosion products or surface deposits can be evaluated for SRB using serial dilutions of the same medium. With a sterile scalpel or tongue depressor, scrape a deposit from a specific-size area (i.e., 1 cm^2) and suspend the material in 10 mL sterile distilled water. If the solid sample has been exposed to seawater or to a salt-containing electrolyte, solids should be suspended in a sterile salt-containing medium of the same approximate salinity. Salinity can be measured with a refractometer. Using a sterile syringe, withdraw 1.0 mL of the suspension and inject it into bottle no. 1 and continue with the dilution series as previously described.

The following test medium is recommended by ASTM[1] Standard D4412 for detection and enumeration of SRB in water or water-formed deposits by the MPN technique. Two media preparations are described. Starkey's Medium A is prepared with reagent-grade water (ASTM D1193Type II), and Medium B is prepared using the water to be sampled. For isolation and enumeration of thermophilic SRB encountered in waters associated with oil and gas production, all broths, dilution blanks, and incubations must be maintained at temperatures of at least 45°C (113°F) and preferably within 5°C (41°F) of the sample environment. Starkey's Medium B is used when the SRB have adapted to atypical nonfreshwater environments. Sensitivity of test methods can be increased by purging dilution blanks and media with nitrogen immediately before use.

[1]American Society for Testing and Materials, 100 Barr Harbor Dr., West Conshohocken, PA 19428

Water deposits and dilutions of these samples are dispensed into tubes of Starkey's Medium (A or B) following five tube MPN procedures. The tubes are sealed with liquid paraffin and incubated at 20°C (68°F) for 21 days. Positive reactions are indicated by the formation of a black precipitate and confirmed using a hydrogen sulfide test reagent.

Starkey's Medium A

Sodium lactate ($C_3H_5NaO_3$)	3.5 g
Ammonium chloride (NH_4Cl)	1.0 g
Dipotassium, hydrogen orthophosphate (K_2HPO_4)	0.5 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	2.0 g
Sodium sulfate (Na_2SO_4)	0.5 g
Calcium chloride ($CaCl \cdot 2H_2O$)	0.1 g
Thioglycollic acid	0.1 g
Ammonium ferrous sulfate or ferrous ammonium sulfate ($(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$)	0.001 g
Water (H_2O)	1.0 L

Starkey's Medium B

The medium is similar to Starkey's Medium A with the following modification: water collected from the sample collection site is used to prepare the medium. The water sample is filtered to remove particulates (1.2 µm membrane filter) and the pH is recorded. After preparing Medium B and prior to dispensing, check and adjust pH if necessary to that of the original water. Filter sterilize medium by passage through 0.2 µm filter and aseptically dispense into presterilized tubes.

Double-strength medium (2X) (as referred to in following procedure) is prepared as above except 500 mL of water are used instead of 1 L. Heat to dissolve and dispense 9 mL of medium per single strength tube and 10 mL per double strength tube. Tubes should be of sufficient capacity to contain 1 mL of inoculum plus 9 mL of single strength medium or 10 mL of inoculum plus 1 mL of 2X medium. The pH of the medium should be 7.0 after autoclave sterilization at 121°C (249°F) for 15 min

Other reagents needed for MPN Test

1) Hydrogen Sulfide Test Reagent:

a) Ferric Chloride Solution ($FeCl_3 \cdot 6H_2O$): Dissolve 13.5 g ferric chloride in a mixture of 250 mL water and 250 mL hydrochloric acid (sp gr 1.19). Store in an airtight amber container. Prepare fresh monthly.

b) *p*-Aminodimethylaniline Dihydrochloride Solution: Dissolve 1 g p-inodimethylaniline dihydro-chloride ($C_8H_{12}N_2 \cdot HCl$) in 500 mL 6 N HCl. Store for up to one month in an amber, airtight container.

2) Liquid Paraffin – Heavy, sterile.

3) Buffered Dilution Water – Stock Phosphate Solution:

Dissolve 34.0 g KH_2PO_4 in 500 mL water, adjust pH to 7.2 with 1 N NaOH.

4) Dissolve 38 g of $MgCl_2$ in 1 L distilled water.

5) Buffered Dilution Water, Working Solution:

Add 1.25 mL stock buffered dilution water and 5 mL $MgCl_2$ solution to 500 mL water. Bring to 1 L with water. Mix well and dispense as 90 mL dilution blanks in screw-capped bottles. Sterilize by autoclaving at 121°C (249°F) for 15 min.

Procedure:

a) Clean and disinfect the area with a 10% dilution of domestic bleach. Allow area to dry.

b) Set out and label five replicate tubes of 10 mL 2X Starkey's Medium, A or B, in the test tube rack.

c) Set out and label five replicate tubes of 10 mL single-strength Starkey's Medium, A or B, for each mL of sample or mL of sample dilution to be tested. Use two sets of five replicate 10 mL tubes, each to contain 1 mL of sample or 1 mL of 1/10 dilution of sample.

d) Prior to sample inoculation, heat tubes of media and dilution blanks in a water bath to 60°C (140°F); cool rapidly to 20°C (68°F) to ensure minimal oxygen levels.

e) Shake sample thoroughly. Make dilutions starting with 10 mL sample into one 90 mL dilution blank.

f) Pipet 10 mL sample into each double-strength broth and 1 mL sample or diluted sample into each set of five single-strength broths.

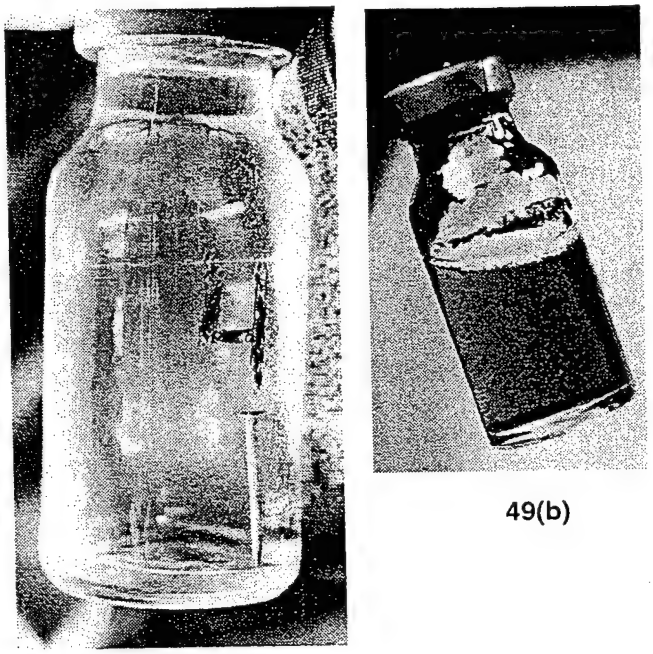
g) Maintain anaerobic conditions by layering with 2 to 3 mL sterile liquid paraffin in each tube.

h) Recap tubes and incubate at 20°C (68°F) 21 days.

Positive reaction is indicated by a black sulfide precipitate. Confirm dubious results by the addition of 0.5 mL ferric chloride reagent followed by 0.5 mL *p*-aminodimethylaniline reagent to the MPN tube. Add reagents to the bottom of the tube using syringe or long Pasteur pipet. A positive reaction, indicated by a blue color, occurs within 10 min if H_2S is present. Compute number of positive findings resulting from multiple-portion decimal dilution planting as the combination of positives and record in terms of MPN/100 mL sample. (See MPN tables, as in references 6 and 14.) Report results as number of SRB/100 mL sample. Unless a large number of sample portions are examined, the precision of the MPN is rather low.

The American Petroleum Institute[2] Recommended Practice (RP-38) for the Enumeration of SRB in Subsurface Injection Waters specifies sodium lactate as the carbon source and an iron nail as a source of iron (Figure 49a). When bacteria are present in the sample, they reduce sulfate in the medium to sulfide, which reacts with iron in solution to produce black ferrous sulfide (Figure 49b). Blackening of the medium over a 28-day period signals the presence of SRB. Williams Brothers Laboratories (Tulsa, OK) and others provide RP-38

[2]American Petroleum Institute, 1220 L St. NW, Washington, DC



49(a) 49(b)

FIGURE 49 - API medium for SRB 28 days after inoculation: (a) negative and (b) positive.

medium in 10 mL quantities in vials containing an iron nail. One mL samples are injected by syringe into medium bottles for tenfold dilutions. It is assumed that only a single living bacterium is required to blacken a bottle.

TABLE 4.2

TEST RESPONSE VS KNOWN NUMBERS: API STRAIN[15]

Actual Count	SRB Numbers Obtained with: RP-38	Agar Deeps	Melt Agar
0	NEG	$>10^5$, NEG	NEG
10^2	10	NEG	NEG
10^3	10^2	NEG	NEG
10^4	10^3-10^4	$\geq 10^4$	17, $>10^3$
10^5	10^4 -10^5	$>10^4$, NEG	NEG
10^6	10^5 -10^6	$>10^5$, NEG	80, 25
10^7	10^7	$>10^5$	3.5×10^2
10^8	10^7 - 10^8	$>10^6$	2×10^3

The simplest test result interpretation is to consider that if one bottle is blackened, the sample contained at least one organism; if two bottles are blackened, the sample contained 10 organisms; three bottles, 100 organisms, etc. The procedure is repeated as many times as necessary to produce a dilution that does not contain organisms as judged by the blackening of the medium. Bioindustrial Technologies (Georgetown, TX) produces a commercial medium that is proprietary, but contains more diverse carbon sources than other commercially available media. The inoculation of the medium and interpretation of results are similar to API procedures.

A solid medium culture technique, "agar deeps," was developed by Biosan Laboratories (Ferndale, MI). The SANI-CHECK™ medium is a modification of API using sodium sulfite as the reducing agent/oxygen scavenger. An agar slant is inoculated by dipping a sterile pipe cleaner into an undiluted sample and inserting into a vial of semi-solid agar. Mineral oil and a CO_2 generating tablet are added to exclude oxygen; the vial is capped, incubated five days, and checked daily for blackening.

A variation of the agar deep technique developed by Nalco Chemical Co. (Chicago, IL) uses melted agar, tryptone as nutrient, and sodium sulfite as an oxygen scavenger. The test procedure involves placing tubes of solidified agar in boiling water and cooling the tubes until the semi-solid agar reaches a temperature of 40-45°C (104-113°F) before adding the sample to the bottom of the tube with a pipet. Tubes are capped tightly and incubated for three days. Results are interpreted by multiplying the number of discrete colonies by the dilution factor. If a tube is generally black with no discrete colonies, the cells are assumed to be too numerous to count and the serial dilutions were not adequate to determine an accurate estimate.

Tatnall evaluated API medium, agar melt, and agar deep techniques for accuracy, specificity, ease of use, time required to obtain results, field vs laboratory use, and obvious drawbacks and limitations, using known quantities of *Desulfovibrio desulfuricans* isolated from oilfield-produced water and unknown samples taken directly from oilfield-produced water with no attempt to concentrate SRB.[15] Among the culturing techniques, RP-38 broth bottles gave accurate numbers at high cell concentrations, but underestimated numbers at low cell concentrations (Table 4.2). Agar deeps generally underestimated known populations. Melt agar tubes performed poorly at all concentrations.

Unlike culturing techniques, direct methods for detecting and quantifying SRB do not require SRB growth. Instead, direct methods measure constitutive properties, including adenosine-5'-phosphosulfate (APS) reductase, hydrogenase, cell-bound antibodies, and DNA.

APS reductase: The APS reductase antibody method (E.I. DuPont de Nemours, Wilmington, DE) detects the presence of APS reductase, an intracellular enzyme found in all SRB. Briefly, cells are washed to remove interfering chemicals, including hydrogen sulfide, and lysed to release APS reductase. The sample is drawn into a transfer pipet containing a porous bead, washed four times, and exposed to a color-developing reagent. In the presence of APS reductase, the bead turns blue in 10 min. The degree of color is directly proportional to the amount of enzyme and roughly proportional to the number of cells from which the enzyme was extracted.

An example procedure using an immunoassay test kit (Conoco Specialty Products, Houston, TX) based on the APS reductase technique is:

1) Rehydrate the chemical lysing reagent.

2) Collect 10 mL liquid sample using a bottle provided with the kit (directions also available for solid samples).

3) Filter and wash sample using bottle filter cap. Invert and shake. Wait 15 s. Then use squeeze bottle to remove all liquid, trapping SRB in filtration matrix. Attach filtration material (and bacteria) in filter cap to wash bottle. Squeeze to remove wash liquid.

4) Remove filter cap with filtration material and attach to lysing reagent bottle (step 1). Dislodge filter cake into lysing fluid and mix well without foaming. Allow two min incubation, and discard filter cap. Snap final filter into place.

5) After lysing incubation, squeeze liquid (should have 10–20 drops) from lysing bottle into immunoreagent vial, mix gently, and incubate for two min.

6) Pour contents of the antibody immunoreagent vial onto test membrane device and wait until liquid is absorbed through the membrane. Squeeze contents of the wash solution tube onto the membrane. After all wash solution is absorbed, squeeze color-developing reagent liquid onto membrane and start timing color development incubation.

7) Interpretation: Refer to temperature guidelines for proper time allowance for color development, then match color after that time period to provided color code to determine SRB concentration in cells mL^{-1}.

Hydrogenase: When sulfates are present, SRB predominate in anaerobic niches within biofilms because some SRB produce hydrogenase enzyme and reduce hydrogen produced by acetogenic bacteria to such a low level that other bacteria cannot compete. Hydrogenase breaks hydrogen molecules down to protons and electrons and transfers the energy to the organism through a series of organometallic molecules in the electron transport system. Not all SRB produce hydrogenase.

A procedure has been developed to quantify hydrogenase from SRB that requires cells to be concentrated by filtration from water samples. Solids, including corrosion product and sludge, can be used without pretreatment. The sample is exposed to an enzyme-extracting solution for 15 min and placed in an anaerobic chamber from which oxygen is removed by hydrogen. The enzyme reacts with excess hydrogen and simultaneously reduces an indicator dye in solution. Hydrogenase activity is established by development of a blue color in less than 4 h. Intensity of blue color is proportional to the rate of hydrogen uptake by the enzyme. The technique does not attempt to estimate specific SRB numbers.

A kit is available from Caproco Ltd. (Edmonton, Alberta, Canada) to detect the presence and activity of hydrogenase using liquid or solid samples. The kit contains reagents, filters, and color charts. Specific directions for biofilm probes, corrosion products, and sludge samples are provided with the kit. This is an example procedure for liquids:

1) Test solution preparation: Pour liquid from vial A into vial B.

2) Sample preparation: Unscrew and separate filter halves, insert clean reusable filter, and reassemble. Collect 10 mL water sample in clean syringe, attach filter to syringe and depress plunger. Remove syringe, fill with air, reconnect filter, and purge filter assembly by forcing syringe full of air through filter. Discard water, open filter, and remove filter paper with forceps. Roll filter paper and insert into vial B. Using new 3 mL syringe and needle, wash filter surface using the liquid/powder mixture as wash fluid. Allow contact for about 15 min, then withdraw all liquid into the 3 mL syringe. Remove needle from the syringe and attach disposable filter. Filter liquid into vial A.

3) Sample analysis: Place vials in holder in base of test chamber. Insert gas generator envelope and anaerobic indicator strip into holder slot. Open marked corner slightly. Do not allow sachet containing palladium catalyst to contact water. Carefully add 10 mL clean freshwater or distilled water to the envelope through opened corner. Place cover and tighten clamp on reaction chamber. Place in 30-35°C (86 to 95°F) area and note sealing time. Check at 30 min intervals for first color development. After 4 h, rate color development based on color chart. To detect weak reactions or confirm negative results, recheck after 24 h and verify anaerobic indicator.

Scott and Davies evaluated test kits based on culture techniques and detection of enzymes in SRB (APS reductase and hydrogenase) for verification and quantification of SRB.[16] Liquid culture techniques included MICKIT III-C™ by Bioindustrial Technologies, Inc., and SRB broth by Petrolite. Solid or semi-solid agar techniques included SANI-CHECK™ SRB Test System by Biosan Laboratories, Inc., and BUG CHECK™ SRB by Angus Chemical Co. (Northbrook, IL). RapidChek™ SRB Detection system by Conoco Specialty Products is an APS reductase assay. The Hydrogenase Test™ by Caproco Ltd. measures cellular hydrogenase.

Test kits were received directly from manufacturers and sent to testing laboratories. All kits were tested with environmental samples and compared with in-house culture methods. In-house culture techniques included media most commonly used by organizations in their own SRB test programs and were not specifically identified except that they did include RP-38 broth and Postgate A and B media. Results (Table 4.3) indicate

TABLE 4.3
RESULTS OF SRB FIELD KIT COMPARISONS[16]

Sample	In-House Method	MICKIT™	Petrolite	BUG CHECK™	SANI-CHECK™	RapidChek™	Hydrogenase™
Detection Limits	$<10^0$	$<10^0$	$<10^0$	$<10^1$	$<10^1$	$<10^3$	NA
Produced Water							
Canada	10^5	$>10^4$	10^4–10^5	BDL	BDL	10^5	Weak
	10^2	$>10^4$	$>10^5$	BDL	$>10^3$	10^5	Strong
	10^4	10^3–10^4	10^2–10^3	BDL	BDL	10^3	BDL
	10^1	10^1–10^2	10^0–10^1	BDL	BDL	BDL	BDL
Texas – Holding Pit	10^2–10^3	10^2–10^3	10^2–10^3		$>10^4$, $>10^4$, $>10^4$	BDL	BDL
	10^3	10^2–10^3	10^1–10^2		$>10^5$, $>10^2$, BDL	10^2–10^3	Very Weak
– Pipeline	10^2	10^1–10^2	10^1–10^2		$>10^2$, $>10^1$, $>10^1$	10^4–10^5	Weak
	10^3–10^4	10^0–10^1	10^2–10^3		$>10^5$, $>10^5$, $>10^4$	10^3–10^4	Very Weak
– FWKO	10^3	10^4–10^5	10^2–10^3	$>10^3$	$>10^3$	BDL	BDL
– Gas Pipe	10^2	10^2–10^3	BDL	$>10^1$	$>10^1$	10^4–10^5	BDL
	10^3	10^3–10^4	10^2–10^3	BDL	BDL	10^5–10^6	Weak
Java	$<10^2$	BDL	BDL	BDL	BDL	BDL	Very Weak
	BDL	BDL	BDL	BDL	BDL	10^4	Very Weak
Power Utility Water							
Lake Erie	10^1–10^2	10^1–10^2	BDL	BDL, BDL, BDL	BDL, BDL, BDL		BDL
	10^2–10^3	10^2–10^3	10^1–10^2	BDL, BDL, $>10^4$	BDL, BDL, $>10^4$		BDL
Lake Huron	10^0–10^1	10^1–10^2	BDL	BDL, BDL, BDL	BDL, BDL, BDL		BDL
	10^1–10^2	10^1–10^2	BDL	BDL, BDL, BDL	BDL, BDL, BDL		BDL
Lake Ontario	10^0–10^1	10^1–10^2	10^0–10^1	BDL, BDL, BDL	BDL, BDL, BDL	BDL	BDL
	10^1–10^2	10^1–10^2	10^1–10^2	BDL, BDL, $>10^3$	BDL, BDL, BDL		BDL
Demineralized	BDL	BDL	BDL	BDL, BDL, BDL	BDL, BDL, BDL	BDL	BDL
	BDL	BDL	BDL	BDL		BDL, BDL	BDL
Process Water							
Recirculated	10^1	10^4–10^5	10^2–10^3	BDL	BDL	10^5–10^6	BDL
Process Filtrate	10^3	10^4–10^5	10^4–10^5	BDL	BDL	10^3	BDL
Seawater							
Java	$>10^3$	10^4–10^5	10^3–10^4	$>10^3$	BDL	10^4–10^5	Weak

BDL – Below Detection Limits

TABLE 4.4

RESULTS OF SRB FIELD KIT COMPARISONS[16]

Sample	In-House Method	MICKIT™	Petrolite	BUG CHECK™	SANI-CHECK™	RapidChek™	Hydrogenase™
Detection Limits	$<10^0$	$<10^0$	$<10^0$	$<10^1$	$<10^1$	$<10^3$	NA
COUPONS							
Java – Produced Water	$<10^2$	BDL	10^0–10^1	BDL	BDL	BDL	Strong
– Seawater	$<10^1$	BDL	10^0–10^1	BDL	BDL	BDL	BDL
	$<10^2$	10^0–10^1	BDL	BDL	BDL	BDL	Very Weak
SLUDGES							
Canada – Produced Water	10^6	10^4	$>10^5$	BDL	BDL	10^5	BDL
	10^6	$>10^4$	$>10^5$	BDL	BDL	10^5	Strong
Texas – Injection	$<10^1$	10^3–10^4	10^0–10^1	BDL	BDL	10^5–10^6	Very Weak
– Rat Hole	BDL	10^1–10^2	10^0–10^1	BDL	BDL	BDL	BDL
Java – Seawater	$<10^2$	BDL	BDL	BDL	BDL	10^3	Very Weak
	$<10^1$	10^1–10^2	10^2–10^3	BDL	BDL	10^4	Weak

BDL – Below Detection Limits

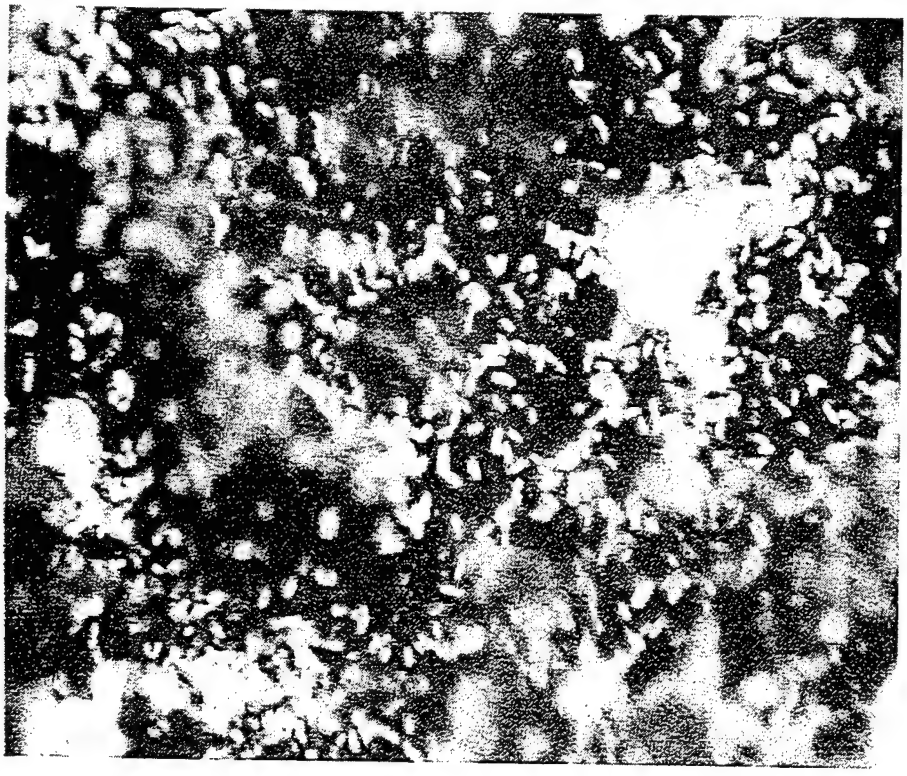


FIGURE 50 - Epifluorescence micrograph of fluorescent antibody reaction for a deposit sample containing SRB (X1,600).[9]

that for produced water samples, the populations estimated with culture techniques were duplicated in most cases with the RapidChek™ method. The hydrogenase assay did not consistently reproduce estimates of the other methods. There was no attempt to quantify the percentage of the natural population that was hydrogenase positive. The same general trends were observed with biofilms removed from coupons and sludge samples (Table 4.4).

Cell-bound antibodies: Field and laboratory epifluorescence cell surface antibody (ECSA) methods for detecting SRB have been developed (Figure 50) based on the use and subsequent detection of specific antibodies, produced in rabbits, that react with SRB cells. A secondary antibody, produced in goats, is then reacted with the primary rabbit antibodies bound to the SRB cells. In the laboratory method, the goat antibodies are linked to a fluorochrome that enables bacterial cells marked with the secondary antibody to be viewed with an epifluorescence microscope.

In the field method, goat antibodies are conjugated with an enzyme, alkaline phosphatase, that can then be reacted with a colorless substrate to produce a visible color proportional to the quantity of SRB present.

Comparisons of the laboratory fluorescent antibody (FA) method to traditional culture techniques for detecting SRB demonstrated that the laboratory FA method is more sensitive to SRB. The detection limit for the laboratory method can be as low as one SRB mL^{-1}. Detection limits for the field test are 10,000 SRB mL^{-1}.

The recommended color reagent used for field testing is unstable at room temperature and tends to bind nonspecifically with antibodies adsorbed directly at active sites on the filter, creating a false positive that may interfere with detection of SRB at levels below 10,000 mL^{-1}. Antigenic structures of marine and terrestrial strains are distinctly different and, therefore, antibodies to either strain do not react with the other. SRB antibodies do not react with non-SRB.

Developers report a poor response of rabbit antibodies developed from pure SRB cultures to mixed populations. Rabbit SRB antibodies generated from fresh SRB strains from Prudhoe Bay, Alaska, as well as terrestrial and marine locations, were found to react better with SRB from natural sources. Antigenic properties of the cell surface change after repeated laboratory culture.

Sample procedure for a cell-bound antibody assay:

1) Spot 10 µL sample on a glass slide and heat fix at 50°C (122°F) for 30 min or pass over a flame. Let cool.
2) Add 10 µL primary antibody preparation directed against organism of interest (serum from rabbits immunized against the specific organism). Let stand at room temperature for 30 min.
3) Wash with phosphate buffered saline (commercially available as hemagglution buffer).
4) Add 10 µL goat anti-rabbit IgG a(ntiserum labeled with fluorescein isothiocyanate. Let stand at room temperature for 30 min.
5) Rinse with phosphate buffered saline. Let dry.
6) Add mounting medium and cover with coverslip.
7) Observe using epifluorescence microscopy at 1,000X magnification. Count number of reactive cells in at least 300 cells or 100 fields. Calculate number of cells mL^{-1} or gram of sample (average number cells/field x 1.1 x 10^5 = number organisms mL^{-1}).

DNA: Gen-Probe Inc. (San Diego, CA) has developed a non-isotopic, semi-quantitative procedure for detecting *Desulfobacter* and *Desulfotomaculum* using DNA probes labeled with an acridinium ester and sensitive to 10^4 organisms mL^{-1}. DNA probes are directed toward ribosomal RNA and consist of four steps:

1) sample handling,
2) binding the probe to the target,
3) removal or destruction of the unbound probe,
4) detection and quantification of the reporter group on the bound probe.

The DNA probes have not been evaluated for field samples and compared with culture techniques.

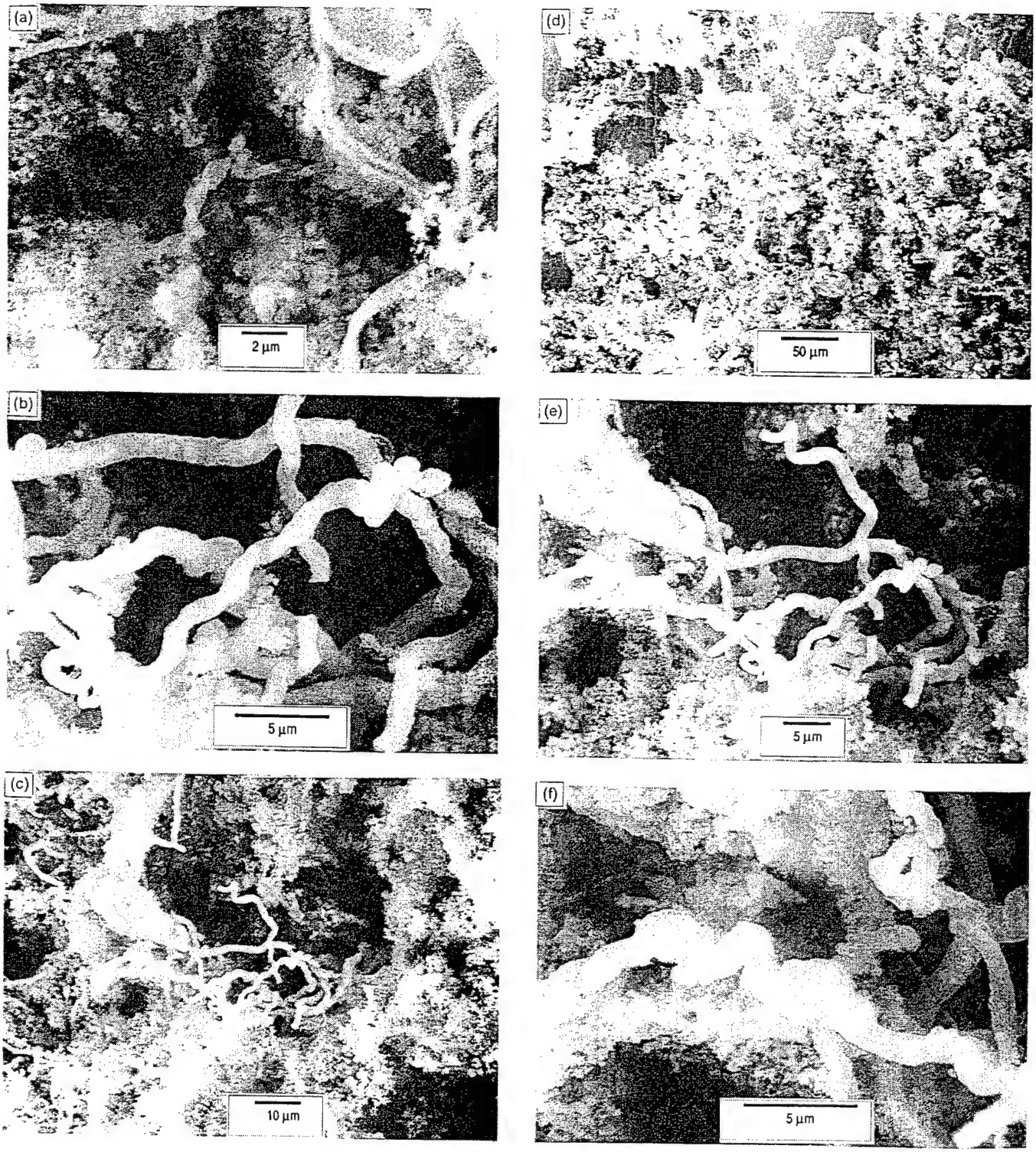


FIGURE 51 (a-f) - *Gallionella* isolated from Figure 53.

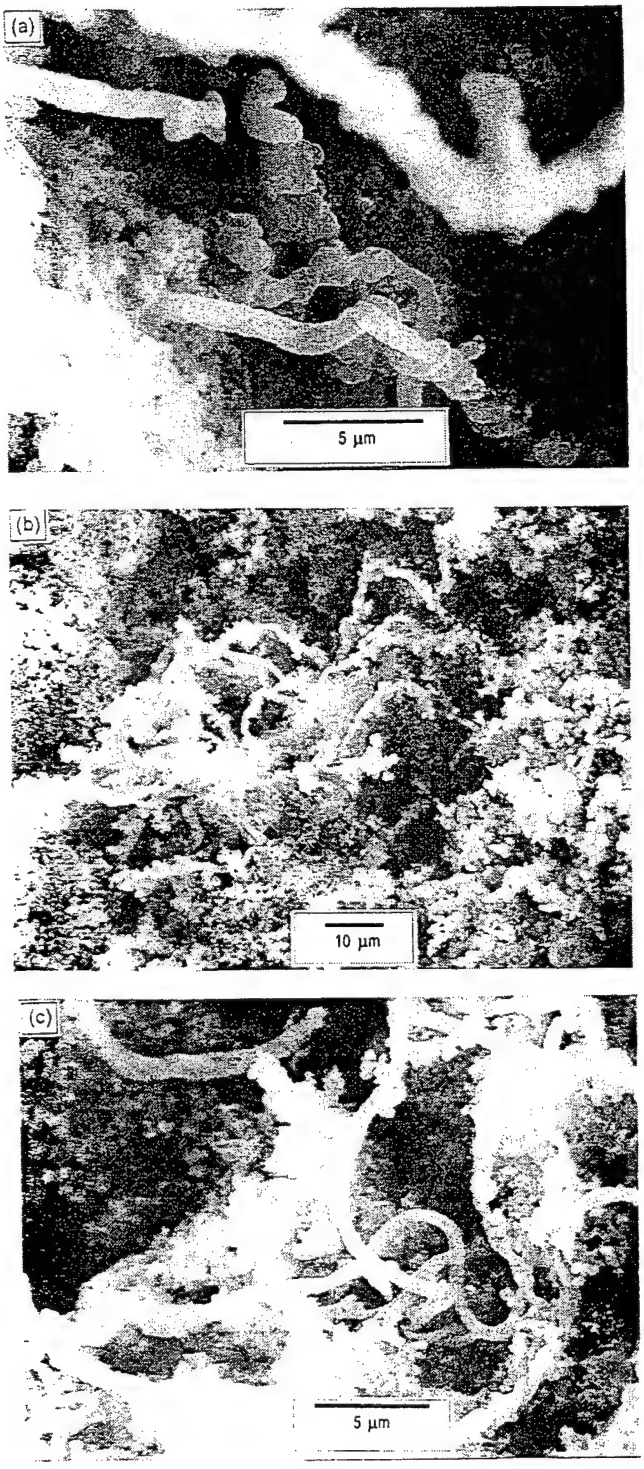


FIGURE 52 (a, b, c) - (above) *Crenothrix* species isolated from Figure 53 (right).

Iron-Depositing Bacteria

The following sections describe indicators for the presence of iron-depositing bacteria or iron-related bacteria (IRB) in solid and liquid samples. Iron-depositing bacteria is a general classification for microorganisms that bind ferric hydroxide and are characterized by the deposition of ferric hydroxide in their mucilaginous sheaths (Figures 51, 52). The process is continuous with growth, and over time, large accumulations of slimy brown deposits occur. The term iron-oxidizing bacteria can be used for those organisms that use Fe^{2+} as a source of energy during autotrophic growth.

The presence of iron-depositing bacteria is suggested when orange, red, and brown slimes with hardened tubercular growths are observed (Figure 53). Underdeposit corrosion occurs under the tubercles. Cloudiness, discoloration, and flocculent growth in the

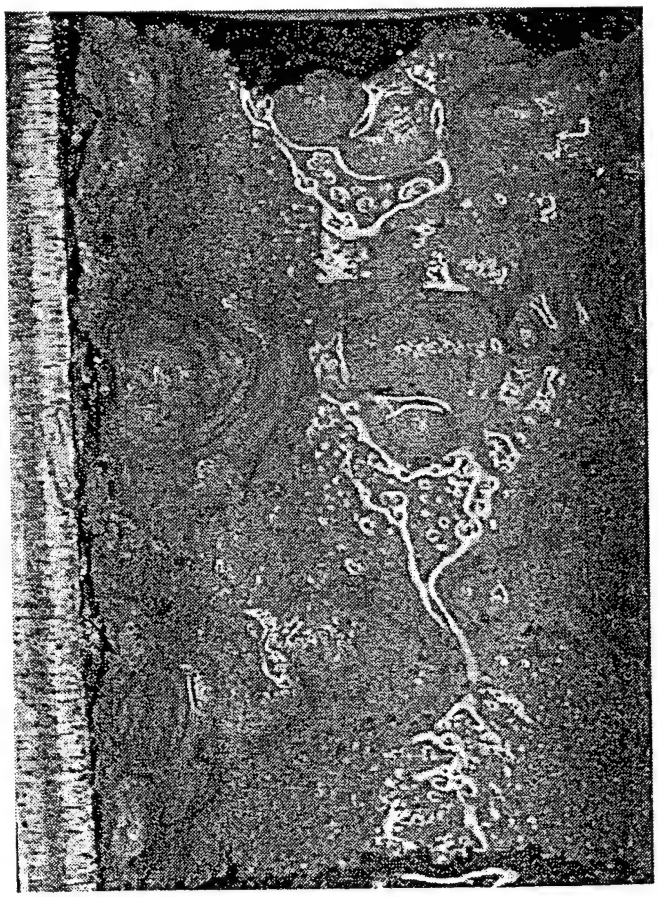


FIGURE 53 - Deposits on galvanized pipe (2X).

electrolyte should be noted. Iron-depositing bacteria may clog water lines, reduce heat transfer, and cause staining. Iron-depositing bacteria generally are filamentous, typically found in freshwater, and are frequently surrounded by a sheath encrusted with iron or manganese, or both.

These genera are classified as iron-depositing bacteria: *Siderocapsa, Gallionella* (*Dioymohelix*), *Sphaerotilus, Crenothrix, Leptothrix,* and *Clonothrix.* Detection of iron-depositing bacteria depends on diagnostic liquid cultures while identification requires microscopic examination. There are no standard techniques for quantification of iron-depositing bacteria. The presence of iron-depositing bacteria within tubercles associated with localized corrosion is considered a positive indication of MIC. Activities of manganese-depositing bacteria produce black deposits on surfaces and similar under-deposit corrosion (Figure 54). Identification and quantification of manganese-depositing bacteria is based on the agar spread plate procedure.

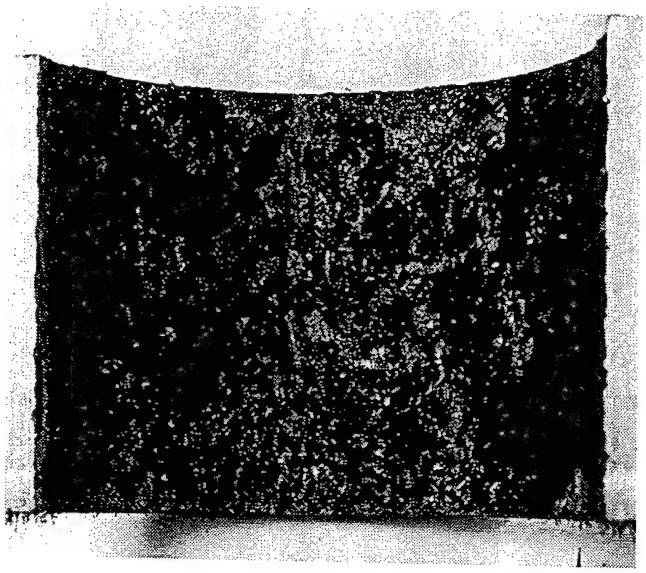


FIGURE 54 - Black manganese dioxide deposits on carbon steel caused by metal-depositing bacteria.

Procedures for identifying iron-depositing bacteria

These procedures can be used to identify iron-depositing bacteria microscopically. Necessary apparatus and materials include:

1) Centrifuge and conical tubes;

2) Microscope and light source with 400 to 1,000X objectives; a dark-field condenser is desirable;

3) Ten mL pipets with an opening 3 to 4 mm in diameter for thick samples, and 1 mL pipets for thin samples;

4) Spatula, small and narrow, for handling thick samples;

5) Membrane filter assembly;

6) Filter paper or blotter;

7) Slides, standard type, 25 x 76 mm (1 x 3 in.) with either plain or frosted end;

8) Cover glasses, round or square type, 19 mm (3.4 in.) in diameter;

9) Ammonium oxalate – Crystal Violet Solution – Mix 2.0 g crystal violet (90% dye content) in 20 mL ethyl alcohol (95%) with a solution of 0.8 g ammonium oxalate monohydrate $(NH_4)_2C_2O_4 \cdot H_2O$) in 80 mL water.

Hydrochloric acid (1 + 4) – Mix 1 volume hydrochloric acid (HCl, sp gr 1.19) with 4 volumes water.

Iodine solution – Dissolve 1 g iodine in a solute containing 2 g potassium iodide (KI) in 10 mL water and dilute the resulting solution to 300 mL with water.

• Collect a 500 mL water sample using a sterile 1 L bottle. The bottle should not be more than half filled because of the oxygen demand of suspended matter; filling the bottle may cause the sample to become anaerobic. Soil, sediment, and mud samples should be collected from the solid-water interface for maximum bacterial populations.

• Transfer solid samples to wide-mouth bottles and add clean, chlorine-free water to cover the deposits. Maintain moisture until examined.

Examine microscopically for presence of iron-depositing bacteria:

• Place a portion of the sample on the slide and apply a cover glass. A spatula or wide-mouth pipet can be used to transfer the sample to the slide. Use a pipet to transfer floc material. Flocs settle to the tip when the pipet is held in a vertical position and are concentrated in the first drop. In the case of very dilute solids or a

water sample, concentrate organisms by centrifuging, pour off the supernatant liquid, and repeat if necessary.

• An alternative procedure is to filter a suitable volume of the dilute solids or entire water sample through a 0.45 µm membrane filter in a filtration assembly (holder, tubing, trap, flasks, and vacuum pump). For this test it is not necessary to sterilize the filter assembly for each sample, but the assembly should be thoroughly cleaned between tests.

• Examine slide under the microscope to determine if encrusted, colorless or colored sheaths are present. Place diluted hydrochloric acid (1 + 4) at one side of the cover glass and draw it underneath by absorbing the liquid at the opposite side with a filter paper or blotter. Continue this procedure until no more yellow ferric chloride is evident in the solution. Take care that the flow of the liquid is not fast, or the sample may be drawn to the absorbent material. This treatment removes iron deposited in the sheaths of the bacteria and exposes cells.

• In a similar manner, rinse the iodine solution under the cover glass until the color of the liquid becomes yellow or the filter paper becomes colored. The iodine stains bacerial cells brown and makes them more easily distinguished.

• Examine slide under a microscope using a high-power, dry objective for the presence of *Sphaerotilus, Crenothrix, Leptothrix,* and *Clonothrix.*

• Prepare a new slide by placing a drop of the sample on a clean slide and allowing it to air-dry. Stain for 1 min with ammonium oxalate-crystal violet solution, wash with distilled water, and allow to dry. Examine the slide under an oil-immersion lens for the presence of *Siderocapsa*, which will appear violet colored.

The following four tests are taken from Cullimore.[17] Incubation at room temperature is recommended as a matter of convenience. The tests do not require specialized equipment and can be easily interpreted.

Rodina test: The Rodina test depends on the ability of some iron-depositing bacteria to generate a red flocculant. Sample water is poured to a depth of 10 cm into a wide-necked flask and allowed to stand overnight. The wide neck generates a large surface area through which oxygen diffuses into the water. Under these conditions, some iron-depositing bacteria form flakes that resemble discolored cotton wool, confirming their presence. (Figure 55).

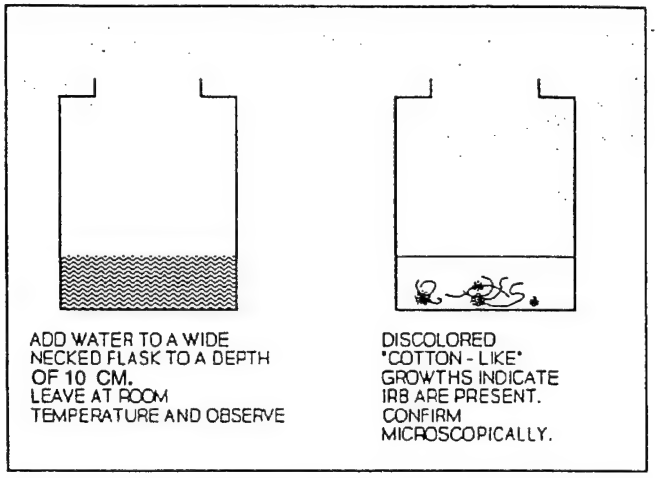


FIGURE 55 - Rodina test.[17]
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Cholodny test: The Cholodny test is based on the fact that some iron-depositing bacteria can be seen if the organisms attach to glass. To conduct the Cholodny test, sample water and accompanying sediment are placed in a jar. A cork with several glass cover slips or slides attached to the underside is floated on the water surface (Figure 56). As the water clears through sedimentation, the glass attached to the cork becomes visible. The development of "rust spots" or "cotton-like accumulations," either on the surface of the sediment or on the glass inserts, within 24 h incubation is indicative of the presence of iron-depositing bacteria. Glass inserts can be removed with the cork from the water, detached, and examined microscopically to identify num bers and types of iron-depositing bacteria.

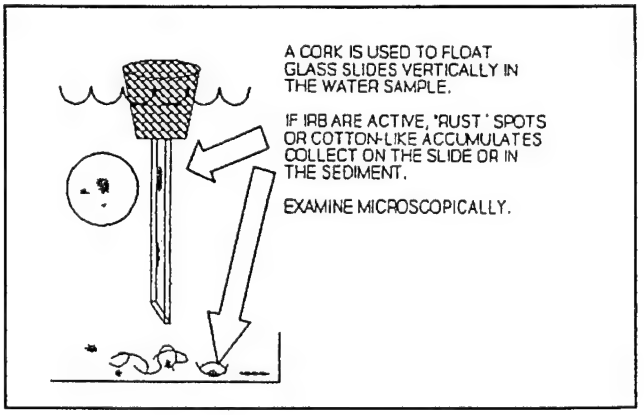

FIGURE 56 - Cholodny test.[17]
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Grainge and Lund test: In this test, iron is added to a water sample in such a way that iron-depositing bacteria activity can be easily identified. An acetone-washed soft steel washer is placed in a conical flask (e.g., Erlenmeyer flask) and an extruded plastic rod (e.g., stirring rod) positioned vertically (Figure 57). Sample water is added to cover the soft steel washer. The end of the plastic rod is exposed to air. After two days, translucent, brownish, string-like growths at the water line around the plastic rod are indicative of the presence of iron-depositing bacteria in the water sample.

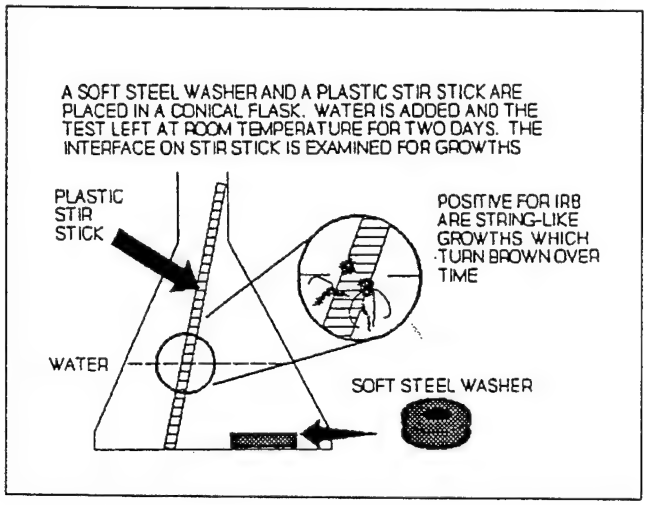

FIGURE 57 - Grainge and Lund test.[17]
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The George Alford test: A non-galvanized iron washer is introduced to a water sample along with a readily available carbon source (ethanol) to stimulate the activity of the iron-depositing bacteria. To test, 150 mL of a water sample and a non-galvanized washer are added to a clean glass container with two drops of ethanol. Cover the glass loosely with aluminum foil (to reduce evaporation) and incubate at room temperature (Figure 58).

After two days, a positive reaction is recognized by either a "fuzzy" growth around the washer or metallic "floaters" in the water. In both cases, iron-depositing bacteria are either actively attaching directly to the source of iron (i.e., the washer) or accumulating the dissolved iron within suspended particulate masses (i.e., the floaters).

Key for identifying iron-depositing bacteria:

I. TRUE BACTERIA:

Capsulated coccoid or short rods
Genus: *Siderocapsa*

Organisms are coccoid or short rods, occurring in groups of 1 to 30 but generally less than 10, surrounded by a mucoid capsule. The deposit surrounding the capsule is rust-brown due to the presence of hydrous ferric oxide.

II. STALKED BACTERIA:

Twisted or straight bands resembling a ribbon or a row of beads. Bacteria are rod-shaped and borne at the top of the stalk.

Genus: *Gallionella* (*Didymohelix*)
(Figure 51 a-f)

The stalks are slender (1 to 3 µm), dichotomously branched, and composed of colloidal hydrous ferric oxide. The bacteria are frequently overlooked, and the stalk is considered as the bacterium.

III. FILAMENTOUS BACTERIA:

A. Not encrusted with iron.
Genus: *Sphaerotilus*

Filaments are attached, colorless, and may show false branching. The cells are rod-shaped or oval, 1.5 to 4 µm

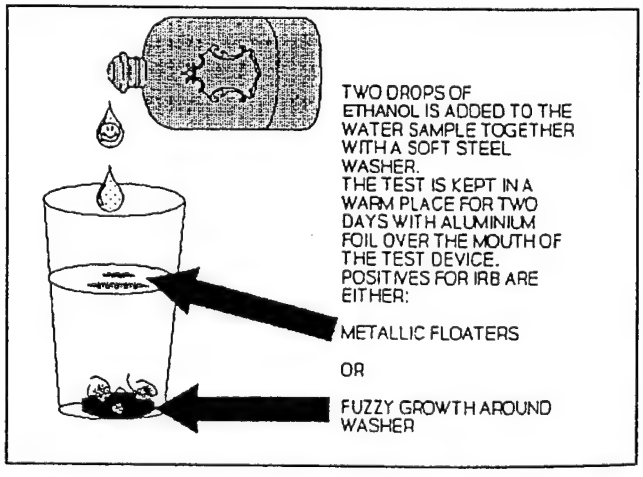

FIGURE 58 - The George Alford test.[17]
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in diameter, surrounded by a firm sheath that is entirely organic and not impregnated with iron.

B. Encrusted with iron.
(1) Not branched:
Genus: *Crenothrix*
(Figure 52 a-c)

Filaments usually are attached to a firm substrate and are differentiated into a base and a tip. The sheath is plainly visible and is thin and colorless at the tip, becoming thick and encrusted with iron oxide at the base. The cells vary from cylindrical to spherical, the diameter being between 2 and 9 µm. Spherical, nonmotile reproductive bodies are formed. False branching may occur due to germination of spores within the sheath.

(2) May be branched:
(a) Cells from 0.5 to 1 µm in diameter.

Filaments contain colorless, cylindrical cells which first have a thin, colorless sheath that later becomes encrusted with iron oxide.

(b) Cells 2 µm or more in diameter.
Genus: *Clonothrix*

Filaments attached show false branching. Sheaths are organic and encrusted with iron hydroxide or manganese, broader at the base and tapered to the tip, varying from 7 to 2 µm. Cells are colorless, cylindrical, 2 x 10 µm. Filaments are colorless when young, becoming dark, yellowish-brown with age. Forms spherical reproductive cells on the short branches of the younger portions of the filaments.

The following staining techniques, adapted from Cullimore[17], are specific for iron-depositing bacteria.

Olanczuk-Neyman method: The Olanczuk-Neyman staining technique requires membrane filtration to concentrate suspended particulates prior to staining.

- Dilute 10, 1, and 0.1 mL aliquots of water sample into 110, 119, and 119.9 mL (respectively) of sterile Ringers solution (see page 34) to create final volumes of 120 mL. Gently mix each dilution.
- Filter each dilution through a 0.45 µm membrane filter and immerse each filter into a 1.0% solution of formalin for a minimum of 20 min.
- Following fixation in formalin, immerse the filter in a 2.0% solution of potassium ferricyanide for 20 min.
- Rinse each filter in 5.0% hydrochloric acid for 3 min.
- Rinse gently with distilled water.
- Cover filter with a 5.0% solution of erythrosin A in a 5.0% phenol-based solution. (Note: 2.0% solution of safranin can be substituted for the erythrosin A.)
- Gently rinse the membrane filter with distilled water to remove stain and air-dry.
- Examine microscopically. Bacterial cells appear red and iron deposits, blue.

Meyers stain: Meyers stain primarily is recommended for use with water samples showing some sign of iron-depositing bacterial contamination (e.g., the water is discolored to a yellow, orange, or brown hue; the water has a distinct colored deposit that may have an indistinct or fuzzy outline).

- Separate cellular material by centrifugation to the extent that a pellet is formed in the base of the centrifuge tube. The precise level of centrifugation will vary since a greater amount of g force (gravity) may be required to compact cells in some waters.
- Remove water carefully with a pipet so as not to disturb the pellet.
- Using a sterilized bacteriological loop, withdraw some of the pellet and smear on a bacteriological slide.
- Air-dry the slide and heat-fix by passing the slide quickly three times through the lower part of a blue flame. Be careful not to overheat the slide. Only enough heat is required to make it warm to the touch.
- Immerse slide in absolute methanol for 15 min.
- While the slide is immersed, bring to boiling point a 1:1 mixture (v/v) of 2% potassium ferricyanide and 5% acetic acid, both as aqueous solutions in distilled water.
- Remove slide from the methanol and immerse in boiling mixture for 2 min.
- Remove slide and allow to cool.
- Gently wash slide with distilled water.
- Cover slide with an aqueous 2% safranin solution

and leave for 10 min.

• Rinse slide with distilled water, air-dry, and examine microscopically.

The Meyers stain is not quantitative but does allow one to verify the presence or absence of iron-depositing bacteria. The technique is not suitable for waters with a low iron-depositing bacteria population.

Leuschow and Mackenthum Membrane Filtration technique: The Leuschow and Mackenthum Membrane Filtration technique can be used with waters having a low number of iron-depositing bacteria. Unlike staining techniques, this procedure involves direct observation of the bacteria on a membrane filter, dried and rendered transparent with immersion oil. The technique is restricted to bacteria with large and/or distinctive structures. Stalked and sheathed iron-depositing bacteria may easily be seen using this technique.

• Filter 100 mL of the water sample through a 0.45 µm membrane filter.

• Remove filter and thoroughly dry in a hot oven.

• Saturate the dried membrane filter with immersion oil.

• Place filter on a bacteriological glass slide and examine under high power (400-600X).

Negative wet mount stain: One major problem with staining techniques is that the "edge" of bacterial cells, particles, sheaths, and stalks may be diffuse and difficult to observe. Additionally, where bacteria are not pigmented or have not accumulated iron, cells may be transparent to light and not easily recognized. Negative stains use a different concept in that the background is stained while bacterial cells remain unstained. Cells stand out as being illuminated zones within a darkened (stained) background. In this case an acidic stain, nigrosin, is used. It does not penetrate or stain bacterial cells. The stain does produce a deposit around the cells, forming a dark background in which bacteria appear as clear, unstained regions that relate to the shape of the cells. Where nigrosin is not available, India ink (25% aqueous solution) may be used.

Negative stains are more satisfactory for waters showing visible signals of possible microbial presence (discoloration and clouding) and have the advantage that all types of bacteria may be observed by this technique. There are a number of mechanisms to obtain the sample for the negative stain. Approximately 0.2 mL of suspension is used in this staining technique. A very turbid water or a centrifuged pellet probably would have too many cells to be clearly definable. A clear water sample may have too few cells to be conveniently observed. In either event, some dilution (into a sterile Ringers solution) or concentration (by passive settlement or centrifugation) may be needed to conveniently view a dispersed microbial mass. Trial and error may be required to find the appropriate dilution or concentration of the water sample.

• Flame sterilize wire inoculating loop: Hold by handle with the loop in a hot blue flame until the loop glows red. Remove loop from flame and allow to cool slightly. One loopful of liquid sample contains one drop.

• Using a sterile inoculating loop, transfer two drops of the sample to a clean glass slide.

• Add two drops of nigrosin and evenly mix the sample with the nigrosin.

• Spread the mixture over the surface of the slide using the narrow flat edge of a second slide held at an angle to the first slide. As the edge is moved along over the surface of the glass slide, the suspension is spread to form a thin film.

• Lower one end of a cover slip over the central area of the film using forceps. Gradually reduce the angle of the cover slip until it is totally in contact with the film, taking care that no air bubbles are entrapped.

• Using high power and oil immersion-assisted microscopy, examine the film through the cover slip. Cells will appear to be either transparent (if there has been no excessive accumulation of iron) or orange to brown (where iron has been accumulated). The background will appear pale.

CHAPTER 5

Chemical Testing

Adenosine Triphosphate (ATP)

Measurement of ATP using firefly luciferin-luciferase is a rapid, sensitive determination of viable microbial biomass. Because ATP is a compound found in all living matter, ATP assays can be used to estimate the total number of viable organisms. ATP, the primary energy donor for life processes, does not exist in association with nonliving detrital material, and the amount of ATP per unit of viable biomass (expressed in weight) is relatively constant. A conversion factor of 2.5×10^{-10} µg ATP/cell has been used to represent the average ATP content of 19 bacterial species.[18] For specific applications the conversion factor should be calculated using actual bacteria isolated. ATP per cell does vary with species and physiological state of the organism. ATP in a number of bacteria (Table 5.1) was extracted by suspending 1 mL washed cells in 5 mL extracting reagent.

The following test procedure is adapted from ASTM Standard D4012-81, "Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water" (revised 1990). It is adequate to measure ATP concentrations normally found in laboratory cultures, natural waters, wastewaters, and in plankton and periphyton samples from waters. Estimation of ATP in biofilms on metal coupons is not recommended because of the interference of heavy metal contamination from the coupon. Biomass in the sample can be determined by direct ATP extraction when cell counts are greater than 20,000 microorganisms mL^{-1}. When cell counts are less than 20,000 microorganisms mL^{-1} the sample may be concentrated using either centrifugation or filtration prior to ATP extraction. Sample sites should correspond as closely as possible to those selected for microbiological sampling so there is maximum clarity for correlation of results. Extraction procedures should be performed immediately after sample collection. Samples may be stored two to three h if temperature and lighting conditions are maintained. If the analysis cannot be performed within that period, the extracted sample may be stored at -20°C (-4°F) for up to six months.

TABLE 5.1
ATP CONTENT IN BACTERIA[19]

Organisms	Total cell/ml ($\times 10^{8}$)	µg ATP/cell ($\times 10^{-10}$)
Aerobacter aerogenes	15	0.28
Bacillus cereus	3	1.1
Bacillus coagulans	8.8	1.7
Bacillus globigii	4.6	5.4
Brevibacterium helvolum	22	0.37
Erwina carotovora	6.4	0.44
Escherichia coli	16	1.0
Flavobacterium arborescens	6.1	1.5
Gaffkya tetragena	10	0.61
Klebsiella pneumoniae	9	5.0
Micrococcus lysodeikticus	14	1.3
Mycobacterium phlei	5.3	1.9
Mycobacterium smegmatis	6.5	8.9
Pseudomonas aeruginosa	33	1.0
Pseudomonas fluorescens	11	3.1
Proteus vulgaris	7.9	1.8
Sarcina lutea	32	0.37
Serratia marcescens	26	1.0
Staphylococcus aureus	23	0.64

1. Apparatus and materials

The following apparatus and materials are required for ATP testing:

- ATP photometer to measure light output;
- Vacuum filtration system (0.45 µm membrane filters);
- Precision syringe, 50 µL (a constant-rate injection attachment is recommended);
- Automatic pipets and disposable tips;
- ATP-free glassware – rinse chemically clean glassware three times with 0.2 N HCl, rinse three times with tris buffer, and rinse three times with ATP-free water;
- Reaction vial, 6 x 49 mm.

2. Reagents

Reagents must be of high purity so that background light emission is held to a minimum. High-viscosity samples may not mix adequately with the reagents upon injection. Poor mixing results in decreased reaction rates and irreproducible results.

ATP Standard Solution – Weigh 119.3 mg crystalline adenosine 5'-triphosphate-disodium salt using ATP-free glassware. Dissolve ATP in 100 mL fresh 0.02 M tris buffer containing 29.2 mg EDTA (Na_2H_2 EDTA • $2H_2O$) and 120 mg $MgSO_4$. Aliquots are stored at -20°C (-4°F) until required.

Extraction Reagent – ATP can be extracted from samples by various reagents and procedures. The most commonly used extracting reagent is boiling tris buffer.

Hydrochloric Acid (17 mL L^{-1}) – Add 17.0 mL HCl (sp gr 1.19) to a 1 L volumetric flask and bring to volume with water.

Tris Buffer (0.02 M) (Tris(Hydroxymethyl) Aminomethane) – Dissolve 2.5 g of the buffer crystals in 1 L deionized water. Bring to pH 7.75 using HCl. Sterilize by autoclaving for 30 min at 121°C (250°F), 15 psi (103 kPa) pressure, and store refrigerated in stoppered flasks.

ATP-free Water – Sterile ATP-free water may be prepared by treatment in a suitable system involving carbon treatment with deionization, filtration, glass distillation, or sterilization by autoclaving and storage under refrigeration in stoppered flasks.

Luciferase/Luciferin Reaction Mixture – This material is commercially available and should be prepared in accordance with the supplier's instructions. Clean glassware must be used. The luciferase/luciferin reaction mixture must be mixed gently without shaking.

Luciferin, a protein found in bioluminescent organisms, produces light in proportion to energy supplied by cellular ATP when oxidized. The enzyme luciferase catalyzes that oxidation. Luciferase can be inhibited or denatured by the presence of heavy metals, high salt concentrations, and organic solvents in the sample. The ATP luciferase reaction also is affected by some phosphate buffers, inorganic salts, and high magnesium concentrations. Other energy-mediating compounds, such as adenosine diphosphate, cytidine-5-triphosphate, and inosine-5-triphosphate, react with luciferin to produce light, but compared to ATP they are usually present only in small amounts and do not constitute a significant source of error.

Overall reaction is shown by:

$$\text{Luciferin (reduced)} + \text{ATP} + O_2 \xrightarrow[\text{luciferase}]{Mg^{2+}} \text{Luciferin (oxidized)} + H_2O + \text{light}$$

When all necessary reactants are present in excess, light emission is directly related to ATP concentration. Reaction kinetics and specificity are dependent on the purity of the enzyme preparation; sensitivity is controlled by luciferin concentration.

Accurate determinations of ATP require quantitative extraction of ATP from the sample. Separate cells from any possible free (extracellular) ATP and other interfering materials by filtration, centrifugation, sample washing, etc. Omit the separation step if the sample is known to be free of soluble ATP or interfering material. After separation, lyse the cell wall to free ATP for subsequent analysis.

Perform three replicate (triplicate) analyses on each sample to ensure efficiency, reliability, and reproducibility.

3. Extraction procedures

Either of the following procedures may be used for the ATP test.

Procedure A — Boiling 0.02 M Tris Filtration Method:

- Filter 100 mL of sample through a 0.45 µm membrane filter.
- Remove filter as soon as the filtration is complete. Do not allow filter to dry. Break the vacuum just as the liquid passes through the filter. Remove the filter and place in 5.0 mL boiling 0.02 M tris buffer.
- Heat for 5 to 10 min at 100°C (121°F) in a water bath.

Procedure B — Boiling 0.02 M Tris Buffer Without Filtration Method:

- Add 1.0 mL sample to approximately 35 mL 0.02 M tris buffer pH 7.75 in a 50 mL Erlenmeyer flask that has reached a temperature of at least 98°C (208°F) in a boiling water bath.
- Maintain boiling temperature for 2 to 4 min.
- Cool to room temperature.
- Transfer to a 50 mL volumetric flask and bring up to volume with 0.02 M tris buffer.

Procedure B should be used with marine waters extracted directly without filtrations. It is necessary to dilute the sample with 0.02 M tris buffer to avoid inhibition of the luminescence reaction by sodium chloride.

4. Standardization curve

It is necessary to prepare a standard curve:

- Pipet 1.0 mL of the standard ATP solution containing 1.0 mg ATP into a 1 L volumetric flask and bring up to volume with 0.02 M tris buffer. Call this Solution A. Solution A will contain 1.0 µg ATP mL^{-1}. Make the following serial dilutions:

1) 1.0 mL Solution A + 9 mL 0.02 M tris = Solution B, Solution B = 1.0×10^{-1} µg ATP mL^{-1}.

2) 1.0 mL Solution A + 99 mL 0.02 M tris = Solution C, Solution C = 1.0×10^{-2} µg ATP mL^{-1}.

3) 1.0 mL Solution C + 9 mL 0.02 M tris = Solution D, Solution D = 1.0×10^{-3} µg ATP mL^{-1}.

Additional dilutions are required for oligotrophic waters. Standards can be prepared, frozen, and thawed as needed. A minimum of three replicate determinations of each of the standard solutions (Solutions A, B, C, and D) should be used to prepare a calibration curve. Standards should be prepared to contain concentrations of ATP at the lower end, upper end, and midway in the range of ATP concentrations that the analyst suspects (or knows) to be present in the samples to be analyzed.

- Determine (triplicate measurements) instrument response to the reagent blank consisting of sterile extractant. Subtract the instrument response to the blank from the response to dilutions of the standard and plot results vs ATP concentration on log-log paper.

5. Sample analysis

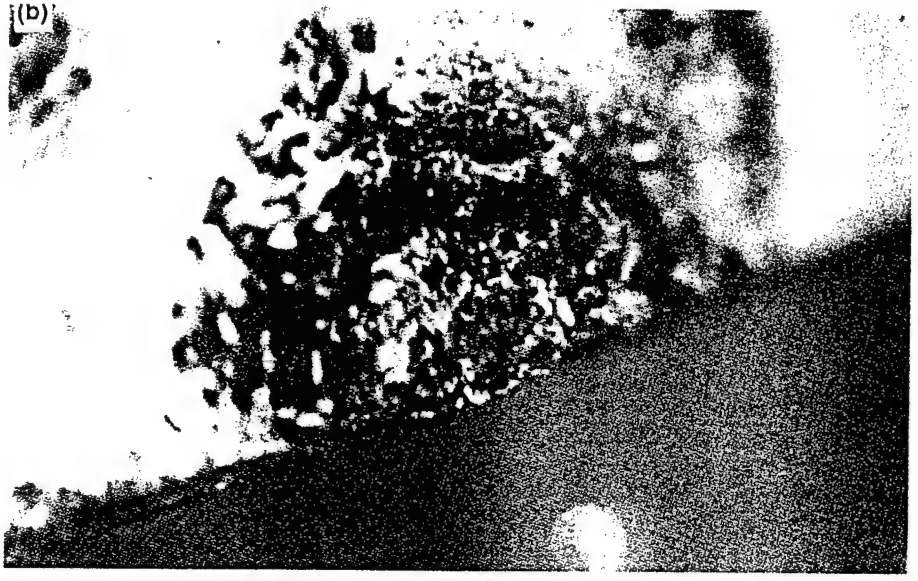
If microorganisms are not concentrated by filtration before ATP is extracted, test the water carrying the microorganisms for the presence of agents that might interfere with the ATP-luciferase reaction by spiking a suitable volume of filtered water that does not contain soluble extracellular ATP.

- Rinse the microlitre syringe three times with 0.2 N HCl by drawing acid into the entire 50 µL then rinse three times with 0.02 M tris buffer solution to neutralize any remaining acid.
- Add sufficient volume of extract to the luciferin-luciferase mixture and measure response with a suitable ATP analyzer.
- Repeat rinse between each sample.
- Convert instrument reading to ATP units mL^{-1} using the standard curve. Account for total sample volume filtered or volume actually analyzed, or both, as appropriate. Data can be expressed in terms of ATP content or approximate cell numbers.

Polysaccharides, Polyphenols, and Proteins

Investigators[20] have demonstrated a relationship between pitting in freshwater copper piping and gelatinous, polysaccharide-containing biofilms (Figure 59). The following sections provide information as to biofilm removal and staining to establish the presence of carbohydrates, polysaccharides, proteins, and macromolecules

FIGURE 59 - (a, b) Gelatinous, polysaccharide-containing biofilm on copper surface. (Courtesy G.G. Geesey, Montana State University).



on copper surfaces. Surface films can be removed from sections of copper tubing or pipes by immersion in 25% nitric acid in a glass Petri dish. Dissolution of the corrosion products detaches organic films within five min. Floating films should be collected using a glass Pasteur pipet and transferred to microscope slides supported on a glass rod, bent into a V shape, placed in a glass Petri dish. Nitric acid carried over with the films is removed by repeated careful irrigation with distilled water from a Pasteur pipet followed by removal of any remaining liquid with finely pointed sections of filter paper. The film material is finally dried on the slide either on a slide warmer or in a 40°C (104°F) oven.

Staining is carried out with slides supported horizontally as described above. Staining solutions are delivered from a Pasteur pipet then drained by tilting the slide, followed by repeated rinsing. Any films that become detached can be retrieved and refixed to the slide by drying. Stained samples normally are mounted in water or dilute glycerol with a cover slip for examination. Alternatively, if a permanent preparation is to be made, the slide-mounted film is dehydrated by flooding sequentially with 50%, 70%, 90%, 95%, and absolute ethanol without allowing the specimens to dry by evaporation. The slide is then flooded with xylene to replace the ethanol and mounted with a glass cover slip.

Periodic Acid-Schiff reagent (PAS) is specific for carbohydrates, and especially polysaccharides:

- Preblock aldehyde groups by treating for 10 min with freshly prepared 0.1 M sodium chlorite in 1% acetic acid.
- Rinse with distilled water for 1 min.
- Oxidize in 1% periodic acid for 10 min and no longer.
- Rinse gently with distilled water for 5 min.
- Stain with Schiff's reagent (commercially available) for 5-10 min.
- Rinse with distilled water until effluent is colorless.
- Examine in water, dilute glycerol, or permanent mount.

A positive reaction shows as a pink-magenta coloration (Figure 60a). Alcian blue/alcian yellow is used to detect anionically substituted polysaccharides:

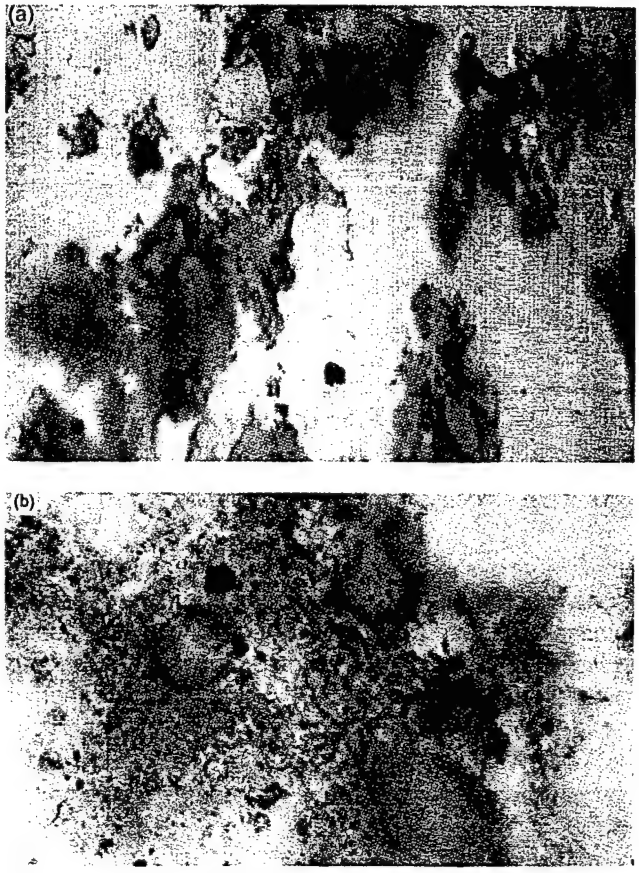


FIGURE 60 - Area stained for polysaccharides using (a) PAS reagent and (b) alcian blue/alcian yellow. (Courtesy A.H.L. Chamberlain, and P. Angell, University of Surrey.)

- Stain for 30-60 min in 0.5% w/v alcian blue solution in 1 M hydrochloric acid (pH 1).
- Wash briefly in 1 M hydrochloric acid.
- Stain for 30-60 min in 0.5% w/v alcian yellow in acetic acid (pH 2.5).
- Wash with dilute acetic acid (pH 2.5).
- Rinse with distilled water.
- Examine in glycerol or permanent mount.

Polysaccharides with sulfate groups will stain blue; those with uronic acid (carboxyl) groups will be yellow; and a mixture of both, green (Figure 60b).

Toluidine blue is used to detect anionically substituted macromolecules and polyphenols:

- Stain for 15 s in 0.05% toluidine blue O in 0.1 M phosphate buffer (pH 5.6).
- Rinse well with distilled water until effluent is colorless.
- Mount in water and observe immediately. Do not use glycerol. Colors are not stable.

In addition to a blue non-specific stain, toluidine blue produces pink-red with acidic macromolecules, and a range of greens from turquoise to grass green with polyphenolic compounds such as humic acids.

Coomassie blue can be used for proteins.

- Stain for 60 min in 0.5% w/v Coomassie blue in a solution of 50 mL methanol + 70 mL glacial acetic acid + 50 mL distilled water.
- Wash well in distilled water.
- Destain overnight at room temperature in a solution of 5% methanol, 7% glacial acetic acid, and 88% distilled water.

Proteins remain stained a deep blue color.

There is no interference by copper ions in the normal staining reactions of the polysaccharides or proteins. Since 25% nitric acid slowly attacks the glycosidic bond, acid-detached films should be transferred from acid as quickly as possible. Ten percent citric acid can be used as a satisfactory alternative to 25% nitric acid, although much longer times are required to detach films from copper surfaces.

Chemical Elements

Some elements in corrosion deposits are indicative of MIC. On carbon steel or cast iron, tubercles or deposits associated with MIC caused by SRB usually contain moderately high levels of phosphorus and sulfur (Figure 61). If iron or manganese-related bacteria have caused localized pitting under a deposit, high levels of chlorine can be expected (Figure 6). Mounds associated with subsurface pitting along stainless steel welds contain very high levels of iron, chlorine, and manganese. Pitting in copper-nickel alloys usually is accompanied by dealloying of the nickel. In 70:30 Cu:Ni alloys exposed in seawater, MIC pits have been depleted of nickel and enriched in copper (Figures 62-65).

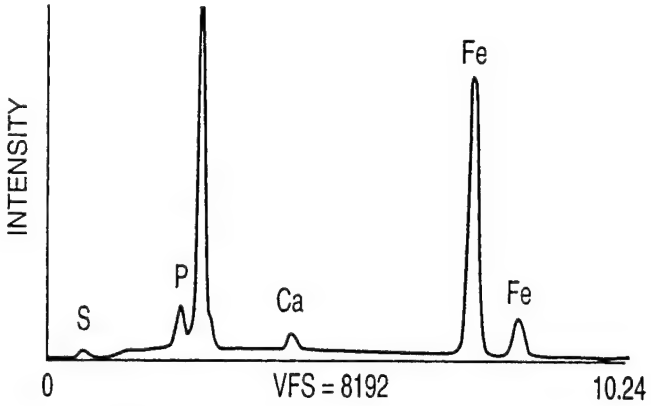


FIGURE 61 - EDS spectrum of tubercular deposits on carbon steel.

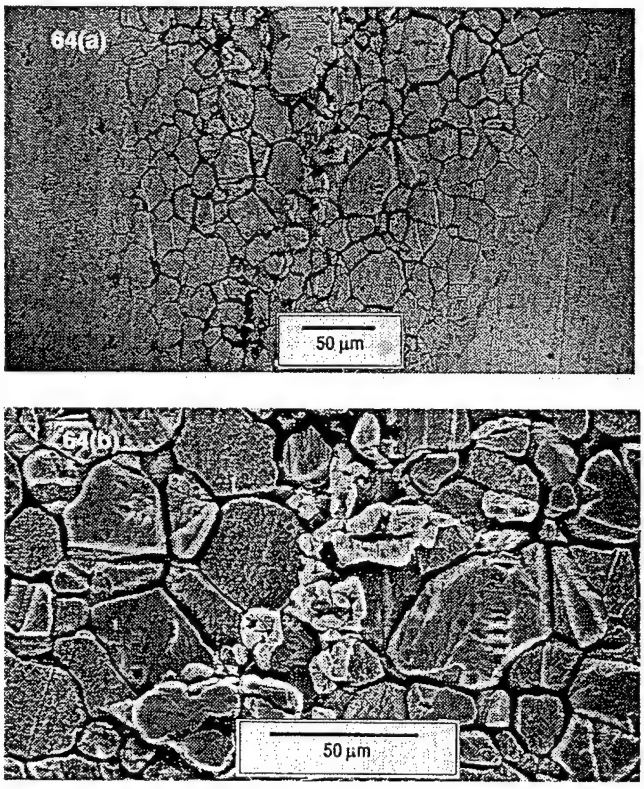


FIGURE 64 (a, b) - Pit interiors on 70:30 Cu:Ni showing localization of copper-rich areas.

FIGURE 62 - Pits on 70:30 Cu:Ni after removal of biofilm, X15 magnification.

FIGURE 63 - X40 magnification of the pits on 70:30 Cu:Ni after removal of biofilm (Figure 62).

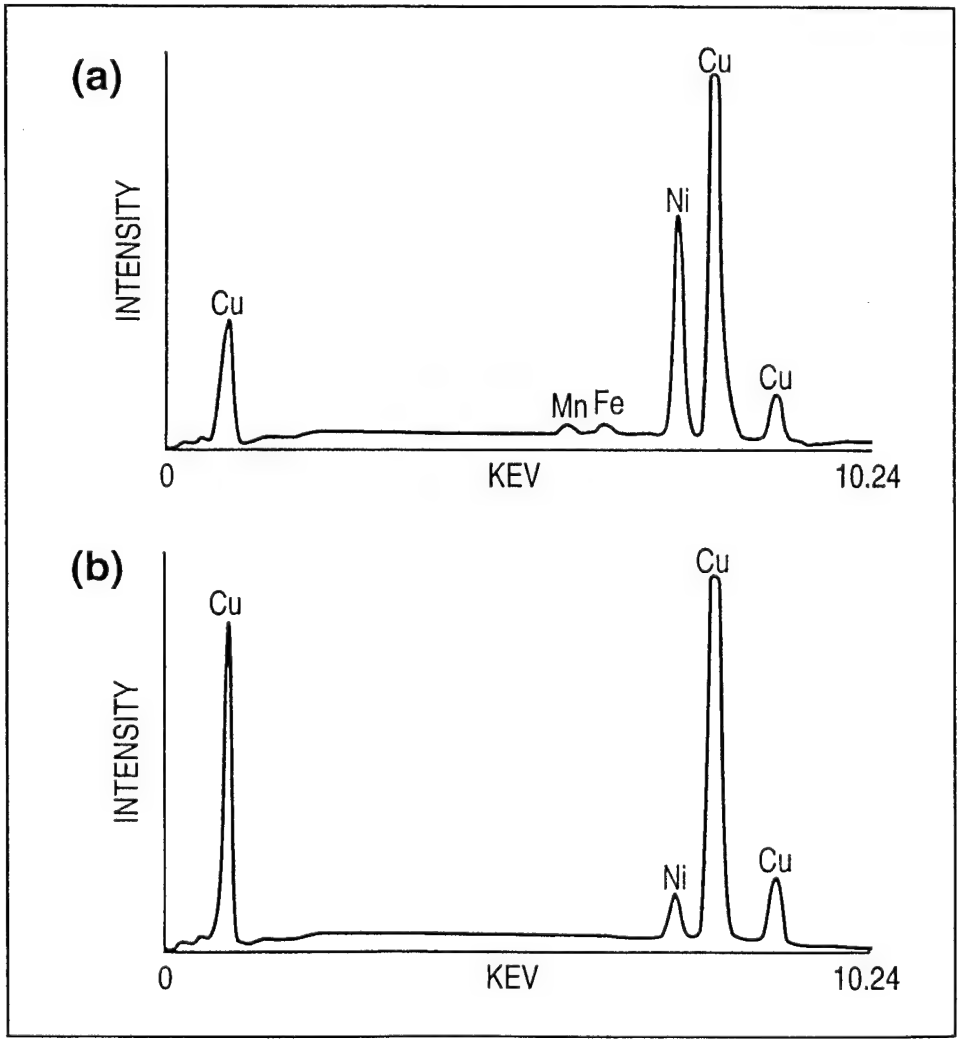


FIGURE 65 - (right) EDS elemental spectra referring to sample in Figures 62 and 63 showing (a) general surface of 70:30 Cu:Ni and (b) denickelification in pit interiors.

The most popular method of analyzing deposits is SEM (See page 31) equipped with energy-dispersive spectroscopy (EDS). Samples can be dried and examined directly or solids can be fixed in glutaraldehyde prior to drying. There are several limitations for EDS surface chemical analyses. Samples for EDS cannot be evaluated after heavy metal coating; therefore, EDS spectra must be collected prior to SEM imaging. It is difficult or impossible to match EDS spectra with exact locations in a SEM image. Collecting EDS spectra at a precise location is not a problem with ESEM because nonconducting samples can be imaged directly, meaning that EDS spectra can be collected from an area being photographed. Shortcomings of SEM/EDS and ESEM/EDS include peak overlap. Peaks for sulfur overlap peaks for molybdenum, and the characteristic peak for manganese coincides with the secondary peak for chromium. Wavelength dispersive spectroscopy can be used to resolve overlapping EDS peaks. It also is impossible to determine the form of an element with EDS. For example, a high sulfur peak may indicate sulfide, sulfate, or elemental sulfur.

Peak heights cannot be used to determine the concentration of elements. Under ideal conditions — polished specimen with external standards — EDS spectra are semiquantitative and can be used only to determine approximate elemental concentrations. Computer programs are available for qualitative, standardless quantitative, semi-quantitative, and quantitative with external standard analyses for elemental analyses. In qualitative analysis the program filters the background, searches for peaks, and identifies elements present in the spectrum. In standardless semi-quantitative analysis a computer program models the background with a matrix correction to quantify EDS spectral elements. A standardless quantitative analysis program filters the spectral background, uses statistical least squares curve fit to determine net peak counts and matrix correction to quantify elements present. Quantitative/standards analyses compares the unknown spectral elements to a material standards reference library and performs statistical best fit tests and matrix correction to determine peak counts.

CHAPTER 6

Electrochemical Testing

Because microorganisms change rates of electrochemical reactions leading to corrosion, electrochemical techniques can be used to evaluate MIC. Electrochemical evaluations can range from mechanistic studies in the laboratory to corrosion monitoring in field applications. Some precautions must be taken with MIC experiments that are not always necessary in conventional corrosion testing.

For example, common methods of accelerating corrosion reactions, such as increasing temperature or concentration of aggressive species, cannot be used. Both factors must be maintained within relatively tight limits; otherwise, unacceptable changes in the microbiology will result.

Conventional anodic or cathodic polarization techniques can produce misleading information because the very high fields produced at the metal surface during polarization are incompatible with viable microorganisms. This problem does not occur with polarization resistance, electrochemical impedance, and electrochemical noise measurements, for which only very small potentials and currents are applied.

MIC is a localized corrosion process that depends on a spatial relationship between microbes and metal substrata. Therefore, electrochemical tests designed to evaluate uniform corrosion may not be appropriate for routine MIC testing.

Most electrochemical techniques give average readings for test electrode surfaces, and it is often not clear whether a measured corrosion current corresponds to uniform corrosion of the entire surface or to localized corrosion of just a few sites on the surface. In the latter case, corrosion rates will be underestimated if measured corrosion loss is not normalized to the area of localized corrosion, a quantity usually unknown.

Background on electrochemical techniques and their use in the study of corrosion phenomena can be found in *Corrosion Testing Made Easy – Electrochemical Test Methods.*[21] In the following sections, electrochemical techniques applied specifically to MIC will be discussed. Equipment needed for the performance of such tests and experimental procedures will be described in detail. ASTM standards will be cited if available. Representative plots and examples for applications will be given for each technique. References documenting such applications will be provided.

Electrochemical techniques covered in this section include measurements of corrosion potential (E_{corr}), reduction-oxidation (redox) potential, polarization resistance (R_p), and polarization curves, including pitting scans. The zero resistance ammeter (ZRA) will be discussed in detail because it forms the basis for several devices designed to detect or quantify MIC. Advantages and disadvantages of each technique will be presented with special emphasis on unique requirements of MIC testing.

Most electrochemical tests require a potentiostat and an electrochemical cell. However, open-circuit potential measurements may be performed using a high-input impedance voltmeter while galvanic current and electrochemical noise measurements may be carried out using a ZRA instead of a potentiostat.

These are required for routine electrochemical testing:

- test electrode or working electrode (Figures 66, 67);
- one or more counter electrodes (Figure 67);
- reference electrode (Figure 67);
- test cell (Figures 67, 68);
- potentiostat (Figure 69); and
- recording device (strip chart or X-Y recorder) (Figure 70).

These are optional for electrochemical testing:

- high impedance voltmeter for monitoring potential and current,
- computer with software program and plotter (replacing recording devices),
- compressed gas tank with nitrogen, argon, or another gas to deaerate solutions,
- constant temperature bath with a thermostat.

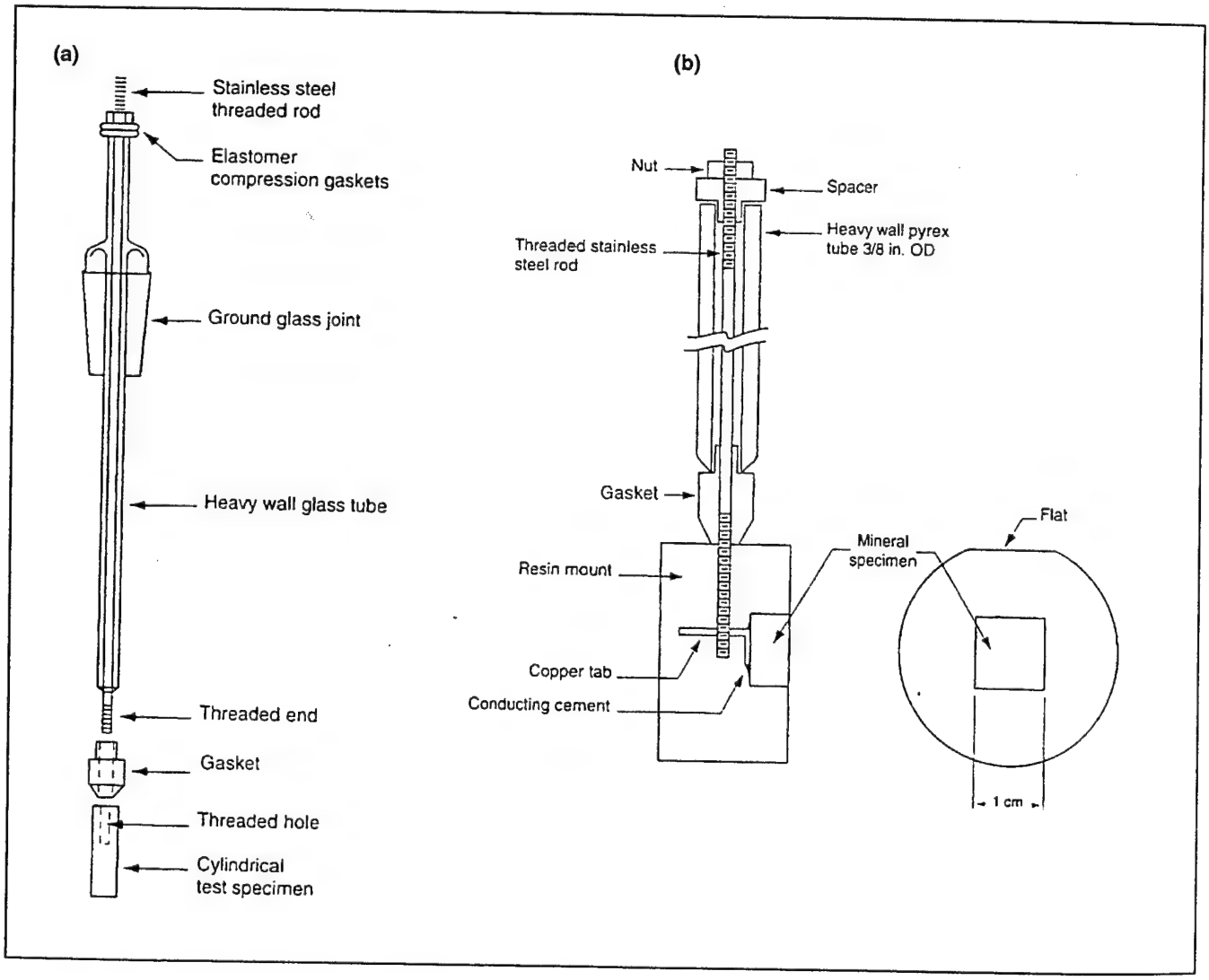


FIGURE 66 - Test electrodes and electrode holders (a) cylindrical and (b) flat disk or sheet electrodes.[22]

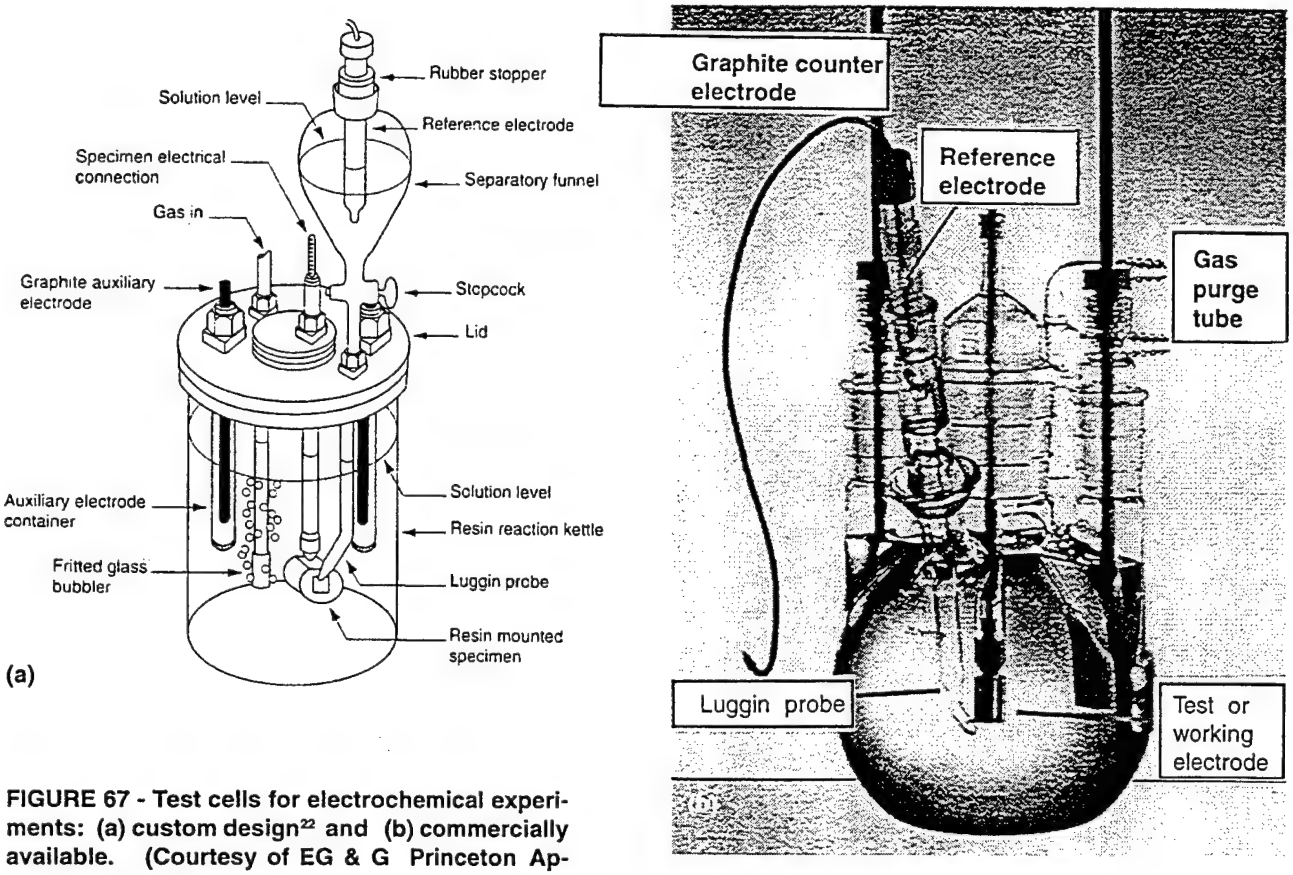


FIGURE 67 - Test cells for electrochemical experiments: (a) custom design[22] and (b) commercially available. (Courtesy of EG & G Princeton Applied Research.)

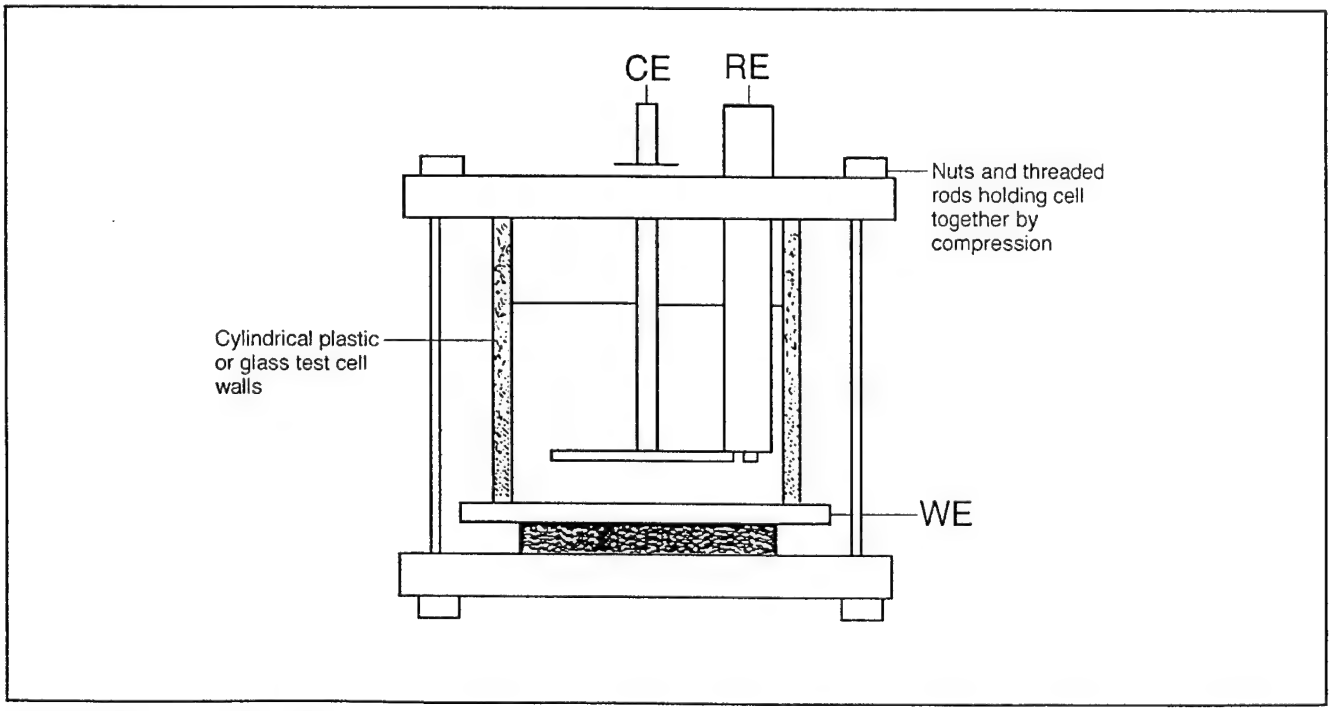


FIGURE 68 - Test cell for flat sheet electrodes. CE = counter electrode WE = working electrode RE = reference electrode.

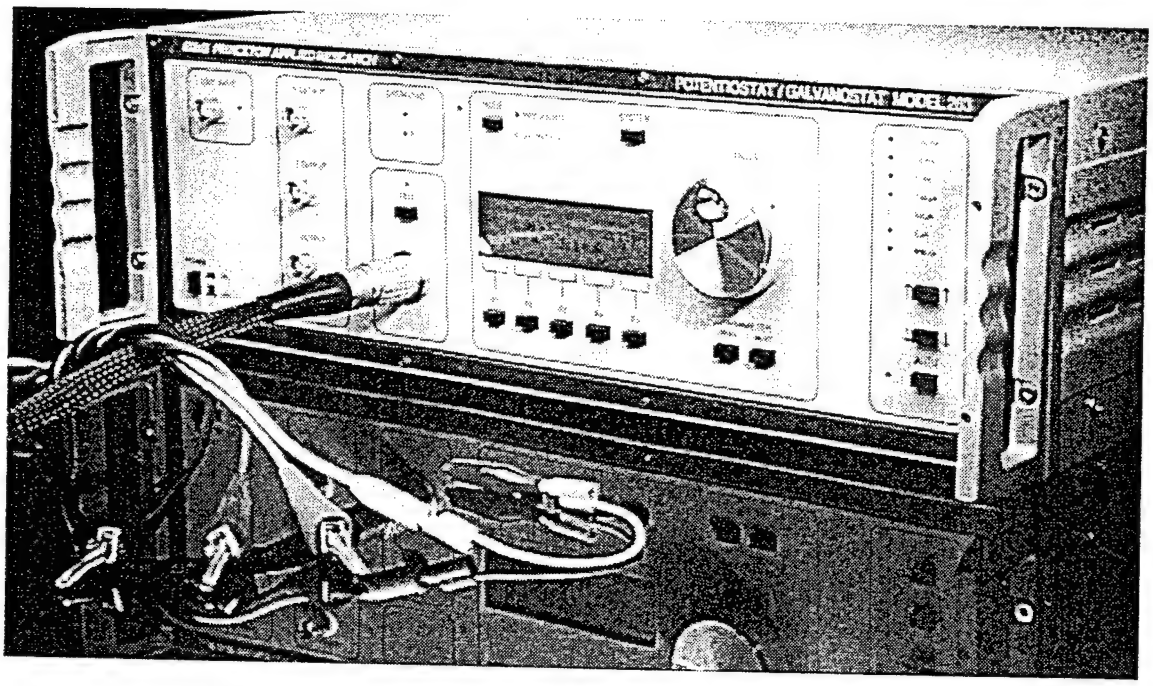


FIGURE 69 - Example of a commercially available potentiostat.
(Courtesy of EG & G Princeton Applied Research.)

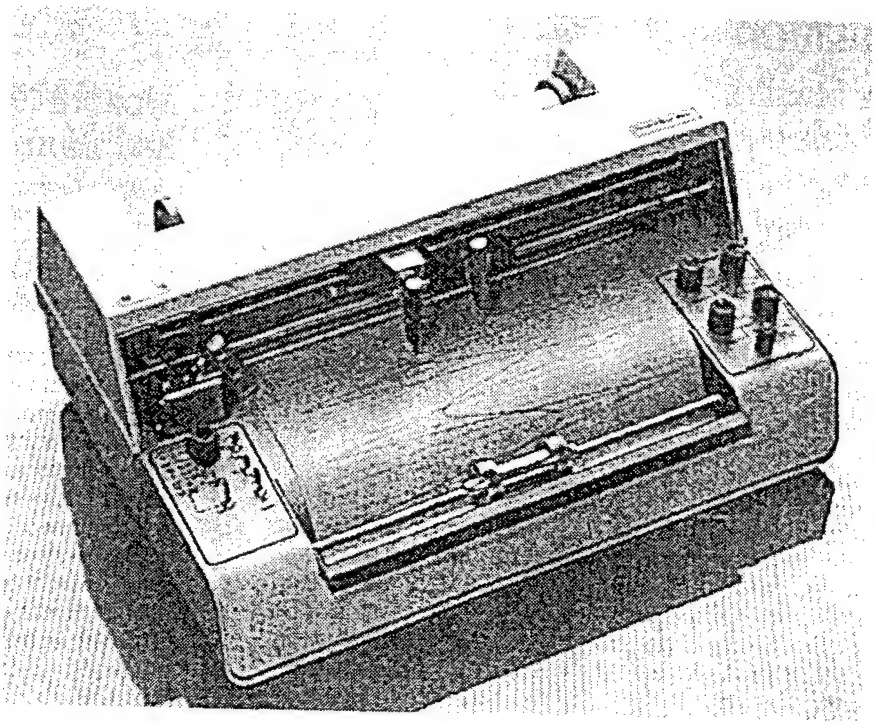


FIGURE 70 - Strip chart recorder.

Abbreviations

These abbreviations will be used consistently throughout this text and are provided for cross referencing. Each will be defined the first time it is used in the text.

A^c/A^a	ratio of cathode area to anode area
B	calculated parameter related to Tafel slopes
b_a	anodic Tafel slope
b_c	cathodic Tafel slope
C	capacitance
E	potential
E_{appl}	applied potential
E_{corr}	open-circuit or corrosion potential
E_{pit}	pitting potential
E_f	final potential
E_s	starting potential
E_{test}	test potential
GPIB	General Purpose Interface Bus
I	current
i	current density
i_{corr}	corrosion current density
I_g	current required to maintain zero potential between dissimilar electrodes in galvanic couple
i_g^a	galvanic current density at anode (I_g/A^a)
i_g^c	galvanic current density at cathode (I_g/A^c)
IR_i	potential difference between dissimilar electrodes in galvanic couple
IR_s	ohmic drop
M	mean
r	rotation speed
R_c	resistance of electrolyte between test and counter electrodes
redox	reduction-oxidation potential
R_m	resistance of measuring resistor
R_p	polarization resistance
$R_{p,exp}$	experimental R_p
R_s	resistance of electrolyte between test electrode and Luggin tip of reference electrode
SCE	saturated calomel reference electrode
SD, σ	standard deviation
SE	standard error
t	time
V_c	cell voltage
V_g	potential of galvanic couple
V_o	output voltage
ZRA	zero resistance ammeter

Basic Instrumentation

Figure 71 shows a typical arrangement for the application of electrochemical techniques in studies of MIC. The potentiostat connected to the test cell is controlled by a computer with suitable software. Data can be printed and/or displayed using software designed for data analysis.[21]

In a potentiostatic experiment, a potential (E_{appl}) is applied between the reference and working electrodes. The working electrode is at virtual ground. The sign of the potential displayed on the potentiostat front panel is usually that of the reference electrode vs ground which is equal in magnitude to, but opposite in sign of, the potential of the working electrode. This often leads to confusion concerning the sign of the measured potential. The instruction manual for each potentiostat should be consulted regarding this point. Current flows between the counter electrode and working electrode and is usually displayed with a positive sign for an anodic current. A simple test can be carried out using a zinc working electrode, which has a very negative corrosion potential (E_{corr}) vs any common reference electrode, such as the saturated calomel electrode (SCE); a negative (cathodic) current is recorded when the applied potential (E_{appl}) is more negative than E_{corr} for the zinc electrode.

Minimum requirements for a potentiostatic test are a test cell (Figures 67, 68) with working, reference, and counter electrode(s); a potentiostat; and recording device(s). Potential and current can be monitored using voltmeters, although this is also possible with built-in instruments in the potentiostat. Most polarization experiments are carried out using software supplied by the instrument manufacturer or from other commercial sources. Experimental data can be stored on floppy disks and analyzed with this software.

The working electrode can have various shapes including cylindrical or flat disk (Fig

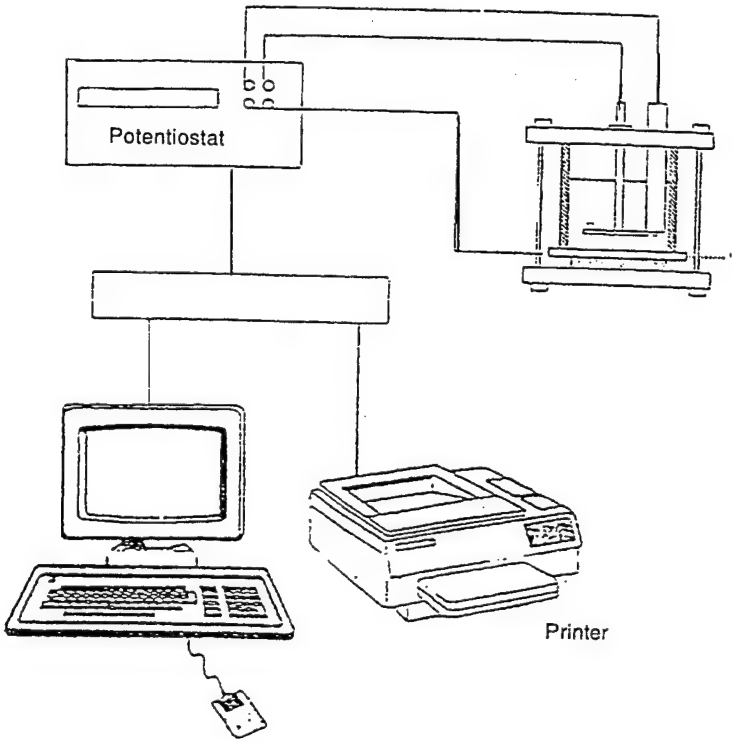


FIGURE 71 - Experimental arrangement for application of electrochemical techniques.

ure 66). The availability of a material as rod or sheet often determines the shape of the electrode. When a cylinder is used, one cylinder end surface is often exposed along with the curved surface. In this case, edge effects can cause varied current distribution along the working electrode surface. Metallurgical properties also may be different for sides and bottoms of cylinders. Fewer problems occur with flat disks. Little attention is usually paid to the size of the electrode, with 1 cm^2 being a common test area. However, a much larger size is useful when very small currents have to be measured. Smaller electrodes are useful when large currents flow in the system because the error due to the ohmic drop (voltage drop between reference and working electrodes due to solution resistance) will be less when small electrodes are used. The working electrode is held in an electrode holder, which incorporates the lead from the electrode to the potentiostat. Details of the construction of electrode holders appear in *Corrosion Testing Made Easy- Electrochemical Test Methods*[21] and will not be repeated here. It is important that only glass, Teflon™, or other nonmetallic components of the electrode holder are exposed in the test solution.

In neutral, aerated solutions, the cathodic reaction (reduction of oxygen) is usually under mass transport control. In this case, the electrochemical experiment should be carried out under controlled

hydrodynamic conditions achieved, for instance, through the use of a rotating disk electrode (Figure 72 a) or rotating cylindrical electrode (Figure 72 b). The rotating cylindrical electrode in which only the sides of the cylinder are exposed to the solution is preferred for corrosion studies since it provides very good current distribution. Polarization curves may be obtained at a constant rotation speed or as a function of rotation speed in studies when details of diffusion-controlled cathodic (reduction) reactions are required.

Counter electrodes should have good electrical conductivity and should not dissolve in the test solution as a result of current flow. Platinum or graphite counter electrodes are used in most experiments. The counter electrode usually is much larger than the working electrode to reduce current density at the counter electrode and resulting buildup of reaction products. To avoid contamination by reaction products such as hydrogen, oxygen, or chlorides, counter electrodes frequently are placed in a separate compartment with a glass frit separating this compartment from the main cell.

The saturated calomel reference electrode (SCE) is the most common reference electrode used in corrosion reaction studies because of stability and commercial availability. The SCE or Hg/Hg_2Cl_2 electrode is a mercury electrode in contact with a specific mercury ion concentration controlled by the concentration (commonly saturated) of potassium chloride (KCl) in saturated mercurous chloride. Care must be taken to avoid contamination of the test electrolyte by leakage of chloride ions (Cl^-) from a SCE when tests are carried out in chloride-free test solutions. A mercury-mercurous oxide (Hg/HgO) reference electrode may be used when Cl^- contamination is not acceptable. Other reference electrodes include the silver/silver chloride (Ag/AgCl) and copper/copper sulfate ($Cu/CuSO_4$) electrodes. It is important to always specify the type of reference electrode used when reporting electrochemical data. A Luggin probe is used to minimize the ohmic drop (IR_s) between the working electrode and the reference electrode (Figure 67).

Several reference electrodes should be maintained together with one master reference electrode that is not used in actual tests. Reference electrodes should be checked against the master reference electrode before use by measuring the potential difference between the two electrodes using a high input impedance voltmeter. The potential difference should be less than

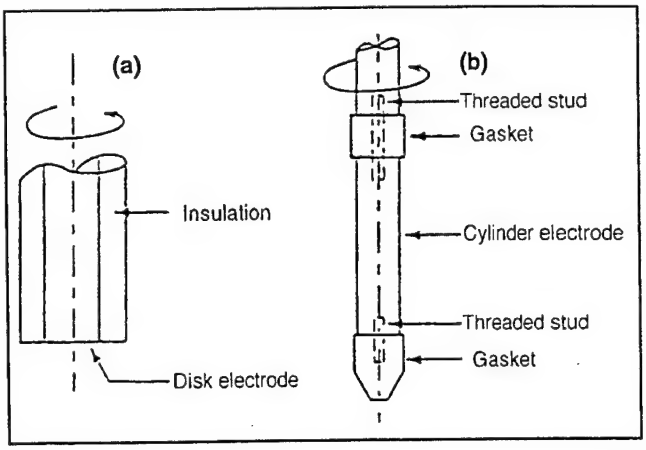


FIGURE 72 - (a) Rotating disk and (b) cylindrical electrodes.[22]

2-3 mV. This precautionary measure is especially important in MIC studies where biofilms can interfere with proper reference electrode functioning. If a larger potential difference is found, the reference electrode should be reconditioned according to manufacturer's instructions. Electrodes still showing a large potential difference after this procedure should be discarded.

Design details of the test cell can be complicated for studies in deaerated solutions with constant temperature control or they can be very simple in tests with oxygenated solutions at room temperature. Three-compartment cells (Figure 67) can be used in the first case while an open beaker gives satisfactory results in the second case. For larger sheet electrodes, such as those used in evaluation of electrodes with polymer coatings or anodic films, the simple cell with the flat sample exposed horizontally as shown in Figure 68 can be used. Important points to consider in assembly of a test cell are correct placement of the Luggin probe for minimization of the ohmic drop and the counter electrode(s) for satisfactory current distribution. Test solutions are deaerated by bubbling high purity nitrogen or argon through the test cell.

Requirements of a potentiostat (Figure 69) are discussed elsewhere in detail.[21] For studies of MIC, the available range of E_{appl} should be ± 2 V, and the current capability should be ± 1 A. Maximum cell voltage should be about 60 V. Voltage outputs should be provided for monitoring potential and current. For most studies of MIC, only small to moderate currents will be observed, and the ohmic drop probably will not cause major

errors in the measurement. Experiments may be conducted under computer control, in which case both the program used to perform the test and the test results can be stored.

Potential and current values usually are displayed on the front panel of the potentiostat. Often, only one of these parameters can be read at a given time. It is sometimes convenient to observe both values on separate voltmeters, especially when meters with a digital display are used. When the electrode potential is measured directly rather than from the potential output of the potentiostat, a high-input impedance voltmeter is required to prevent current flow and polarization of the working and reference electrodes.

Potentials can be recorded on a strip chart recorder (Figure 70) using the voltmeter output. Input impedance of the recorder is not important in this case. However, direct connection of a recorder to the reference electrode and working electrode should be avoided since the input impedance of most recorders is quite low. Recording current vs time curves at a fixed E_{appl} requires a strip chart recorder. Current output, provided in the potentiostat as voltage vs ground, is connected to the recorder with appropriate settings of current range on the potentiostat and voltage range on the recorder. A strip chart or an X-Y recorder can be used to record current vs time curves when stepped potentials are applied in the anodic or cathodic direction. When an X-Y recorder is used, the current output is connected to the Y-axis and the X-axis is set to a time scale appropriate for the time used for each potential step.

It is convenient and time-saving to use a software program to record polarization data. Most commercial software programs contain a number of electrochemical tests and require only a few inputs to be provided by the user. Default values usually are included in the program, and the user can input desired parameters. For current vs time measurements at E_{appl}, inputs will be E_{appl} and time. Additional inputs are software specific. Current vs time curves will be displayed on the computer screen with a current scale selected by the program.

After the test is completed, current vs time curves can be reformatted into more convenient scales. Steady-state current vs E_{appl} techniques, in which the current is measured as a function of time at each E_{appl}, require additional inputs, including starting and final potentials, potential step height, and time. Sometimes the test electrode is held at a preconditioning potential for a certain period before the test is started. A constant temperature bath or a heating/cooling jacket can be placed around the test cell to carefully control temperatures.

Potentiostatic Techniques

Experimental techniques described in this section and in the following Potentiodynamic Techniques sections differ in signal magnitude applied to the working electrode. When measuring E_{corr} vs time, no external signal is applied. In a polarization resistance (R_p) test, a signal is applied that is small enough to ensure a linear relation between potential and current. Since no, or only small, signals are applied, E_{corr} and R_p tests are essentially non-destructive, and many tests can be performed with the same working electrode to evaluate effects of exposure time or other test parameters. Polarization curves usually are recorded over a wide potential range. Since large potential and currents are applied, the working electrode surface can be altered and the test usually cannot be repeated with the same working electrode.

Two commonly used potentiostatic techniques involve measuring current vs time at a controlled value of E_{appl} and steady-state current vs E_{appl}. Experimental procedures for the two tests require the same types of electrodes and test cells. In measurements of steady-state current vs E_{appl}, a series of potential steps is applied, requiring changes of E_{appl} "by hand," and periodic adjustment of the sensitivity of the current-measuring device in the potentiostat and the current scale of the recorder.

The procedure is greatly simplified if appropriate software is used and the experiment is carried out under computer control. Current vs time at E_{appl} data provides information about the time dependence of the rate of an electrochemical reaction at a given potential. This test is useful in MIC studies to determine the effect of additives to the test solution on polarization behavior or initiation time of localized corrosion. Data are used to construct a polarization curve, usually plotted as E_{appl} vs the logarithm of the measured current density (i). Polarization curves also can be obtained with the

potentiodynamic technique discussed below.

ASTM G5, "Standard Reference Test Method for Making Potentiostatic and Potentiodynamic Anodic Polarization Measurements," is used internationally. The test method describes procedures for checking experimental techniques and instrumentation. If followed using ASTM type 430 stainless steel (UNS S43000) in 1.0 N sulfuric acid (H_2SO_4), the method provides the reproducible polarization curves shown in ASTM G5-94. If discrepancies are found, the experiment has not been performed correctly or the equipment is not working correctly.

A complete polarization curve consists of cathodic and anodic parts. The cathodic portion of the polarization curve contains information concerning the kinetics of the reduction reaction(s) for a particular system. Depending on the solution composition, a mass-transport controlled region can be reached at negative potentials where the reaction rate depends only on solution composition and hydrodynamic conditions. Features of the anodic part of the polarization curve strongly depend on the metal/electrolyte system. Usually, a charge transfer-controlled region occurs at potentials close to E_{corr}. Some metals and alloys such as stainless steels may be in an active state close to E_{corr}, but then will show an active-passive transition followed by a passive region and the region of oxygen evolution at highest applied potentials. For those metals susceptible to localized corrosion, a large increase in current will occur in the passive region when the pitting potential (E_{pit}) has been exceeded. ASTM G3-89, "Standard Practice for Conventions Applicable to Electrochemical Measurements in Corrosion Testing," provides conventions for reporting and displaying electrochemical data and contains theoretical and experimental polarization curves.

In general, only qualitative information, such as changes in the polarization characteristics in the passive region or changes of E_{pit} due to the presence of microorganisms, is extracted from a full polarization curve. However, it is possible to determine quantitative information related to corrosion reaction kinetics and corrosion rate.

The relationship between E_{appl} and i in the Tafel region is given by:

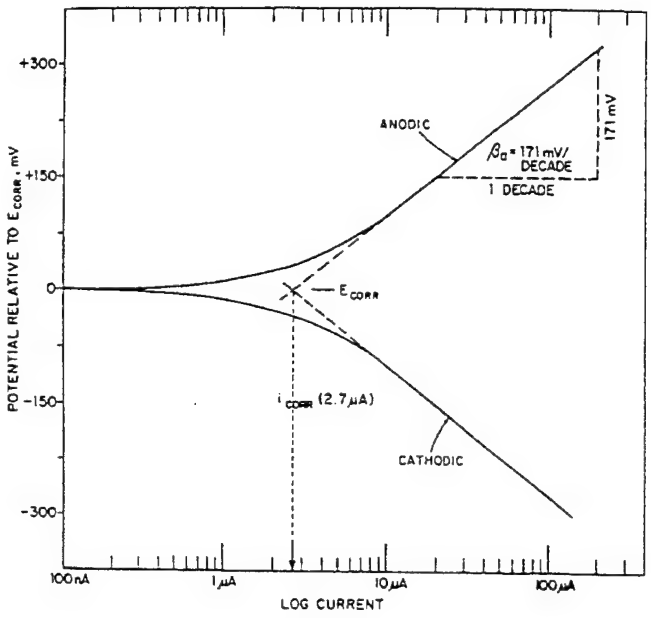


FIGURE 73 - Schematic Tafel plot. (Courtesy of EG & G Princeton Applied Research, Princeton, NJ)

$$E_{appl} = E_{corr} + b_a \log (i/i_{corr}) \qquad \textbf{(7a)}$$

for the anodic polarization curve, and

$$E_{appl} = E_{corr} - b_c \log (i/i_{corr}) \qquad \textbf{(7b)}$$

for the cathodic polarization curve.

Plots of E_{appl} vs log i are called Tafel plots (Figure 73). In Equations 7a and 7b, the anodic Tafel slope (b_a) and the cathodic Tafel slope (b_c) are positive numbers. Corrosion current density (i_{corr}) can be determined according to Equation 7 (a and b) by extrapolation of the Tafel lines to $E_{appl} = E_{corr}$, where $i = i_{corr}$. This method of obtaining i_{corr} is called the Tafel extrapolation method. It is possible to determine i_{corr} from either anodic or cathodic Tafel plots or from the intersection of both plots. It is advantageous to use computer software to record polarization curves and analyze experimental data in terms of b_a, b_c, and i_{corr}. From the numerical values of b_a and b_c, conclusions concerning the rate-determining step in the reaction mechanism can be made. From i_{corr}, the corrosion rate can be calculated using Faraday's Law.[23]

The following calculations are adapted from EG & G Princeton Applied Research Model 350A Corrosion Measurement System Manual (1980).

According to Faraday's Law:

$$Q = \frac{nFW}{M}$$

where Q = coulombs
n = number of electrons involved in the electrochemical reaction
F = the Faraday (96,487 coulombs)
W = weight loss of electroactive species
M = molecular weight of electroactive species

From Faraday's Law, $W = QM/nF$

Since equivalent weight (EW) = M/n

$$W = \frac{Q \times EW}{F}$$

and since Q = It from Faraday's Law

$$W = \frac{It\ (EW)}{F}$$

rearranging gives

$$W/t = \frac{I\ (EW)}{F}$$

W/t is the corrosion rate (CR) in grams/second. It is convenient and traditional to express CR as micrometers per year ($\mu m\ y^{-1}$). These units provide an indication of penetration, assuming uniform corrosion.

Dividing the above equation by the electrode area (A) and the density (d) gives

$$CR\ (cm\ s^{-1}) = I(EW)/dFA$$

After converting seconds to years, centimeters to micrometers, and the Faraday (amp-sec/eq) to microamps, this becomes

$$CR\ (\mu m\ y^{-1}) = \frac{I(EW) \times 31.5 \times 10^6 \times 10^3}{dFA \times 2.5 \times 10^6}$$

Expressing the terms I/A as current density and combining all constants gives the following equation that can be used to calculate corrosion rate directly from i_{corr}.

$$CR\ (\mu m\ y^{-1}) = \frac{3.27\ i_{corr}(EW)}{d}$$

where i_{corr} = corrosion current density, $\mu A\ cm^{-2}$
EW = equivalent weight of the corroding species, g
d = density of the corroding species, $g\ cm^{-3}$

(Note: A helpful rule of thumb to electrochemists: 1.0: $\mu A\ cm^{-2}$ = approximately 10 $\mu m\ y^{-1}$.)

ASTM G 102-89, "Standard Practice for Calculation of Corrosion Rates and Related Information from Electrochemical Measurements," also provides guidance in converting results of electrochemical measurements to rates of uniform corrosion.

The equivalent circuit for a three-electrode cell is shown in Figure 74, where R_p is the polarization resistance of the test electrode and C is its capacitance. R_s is the resistance of the electrolyte between the surface of the test electrode and the tip of the Luggin probe containing the reference electrode, and R_c is the resistance of the electrolyte between the test electrode and the counter electrode. E_{appl} appears across $R_p + R_s$ and always includes an ohmic drop (IR_s), where I is the current flowing as a result of polarization. IR_s is an error in the measurement because E_{appl} set at the potentiostat will differ from the true electrode potential by IR_s. In many cases, where electrolytes with reasonable conductivity are used and IR_s is small compared to E_{appl}, this error can be neglected. In other cases, it can be eliminated by the positive feedback or interrupter techniques available in most modern potentiostats.

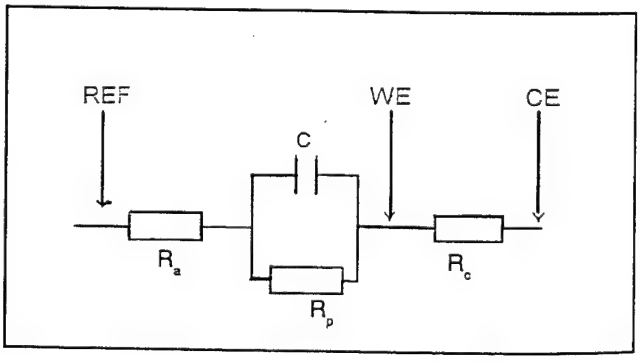


FIGURE 74 - Dummy cell for testing potentiostat.

Potentiostatic Current vs Time at Applied Potential

The experimental procedure for potentiostatic current vs time at E_{appl} measurements requires some electrode pretreatment. Working, counter, and reference electrodes have to be prepared or checked before the test is started. The procedure for checking reference electrodes has been described. Usually, counter electrodes only require degreasing to remove organic matter, oil, and grease. However, if deposits of corrosion products or other solution components from a previous test are detected, a descaling process and/or polishing should precede the degreasing step.[21]

Pretreatment of the working electrode also starts with a degreasing process. Immersion in hot hexanes for several minutes produces satisfactory results. The next step consists of a polishing procedure unless the as-received electrode is to be used without disturbance of the surface. If necessary, very smooth surfaces can be obtained by a final polish with diamond paste or by electropolishing. Cylindrical electrodes can be mounted on a suitable stud and placed into a drill press for polishing. Additional treatments may include degreasing and etching in a pickling or milling solution to remove surface oxides and/or to produce a smooth surface.

The test cell and glassware need to be cleaned periodically with appropriate solutions to remove organic matter and deposits from previous tests. When the test is to be performed in an oxygen-free environment, the electrolyte is first deaerated in the absence of the working electrode by plugging the port for the working electrode with a stopper and purging as necessary. Gas purging should be continued throughout the test. If the test is to be carried out at a constant temperature, the cell needs to be placed in a constant temperature bath or heating jacket. After the constant test temperature is reached, the working electrode can be inserted.

The potentiostat needs to be tested periodically and at any time when problems with the measurement occur. This can be done using a dummy cell (Figure 74). Suitable values are R_s = 10 ohm, R_p = 1,000 ohm, R_c = 100 ohm, and C = 10 µF. Precision resistors and capacitors (1%) must be used. The leads from the potentiostat should be connected to the dummy cell as in Figure 74. If a potential of +1,000 mV is applied by the potentiostat, the current reading should be:

$$= 1{,}000 \text{ mV} / (R_s + R_p) = 1{,}000 \text{ mV} / 1{,}010 \text{ ohm} = 990 \text{ µA.} \quad (8)$$

The potential should then be reversed (-1,000 mV) and the same current with a negative sign should be registered. This procedure can be repeated with different potentials, i.e., 100 mV and 10 mV, to test the potentiostat at different current levels. The sensitivity range at which current is measured also can be changed to further check the unit.

It also is possible to determine the voltage drop across $R_s + R_p$, which should be equal to the applied voltage, and that across $R_s + R_p + R_c$, which corresponds to the cell voltage (V_c) given by

$$V_c = I (R_s + R_p + R_c) = 1{,}099 \text{ mV} \quad (9)$$

The low (or grounded) lead of the voltmeter has to be connected to the same point on the dummy cell as the working electrode lead. If deviations of measured potentials or currents from the correct values exceed 2%, the unit needs to be calibrated before tests are performed.

When the test cell has been assembled, electrodes can be connected to the potentiostat (Figure 71). Sometimes electrode leads are color coded for identification. In most cases, E_{corr} is monitored before starting tests by observing the potential on the built-in meter or external voltmeter. When E_{corr} has reached a reasonably stable value, the potential can be applied and the corresponding current measured. Alternatively, a preconditioning potential can be applied for a certain time or until the current at this potential becomes constant.

The test is started by applying the test potential (E_{test}) and monitoring current. A relatively high current scale needs to be used first to avoid potentiostat overload. A few seconds after E_{test} has been applied, the current scale can be reduced stepwise until a scale is reached for which the current output voltage is between 0 and 1 V. It is convenient to leave the potential scale on the strip-chart recorder fixed at 1 V full scale unless very accurate resolution of the current measurement is desired. The time axis of the recorder needs to be set to a value reasonable for the time for which the potential step is applied. If this time is only a few minutes, a fast speed is needed; otherwise, the chart speed can be much slower.

Often, a series of potential steps is applied to the same test electrode. This would be the case in a test in

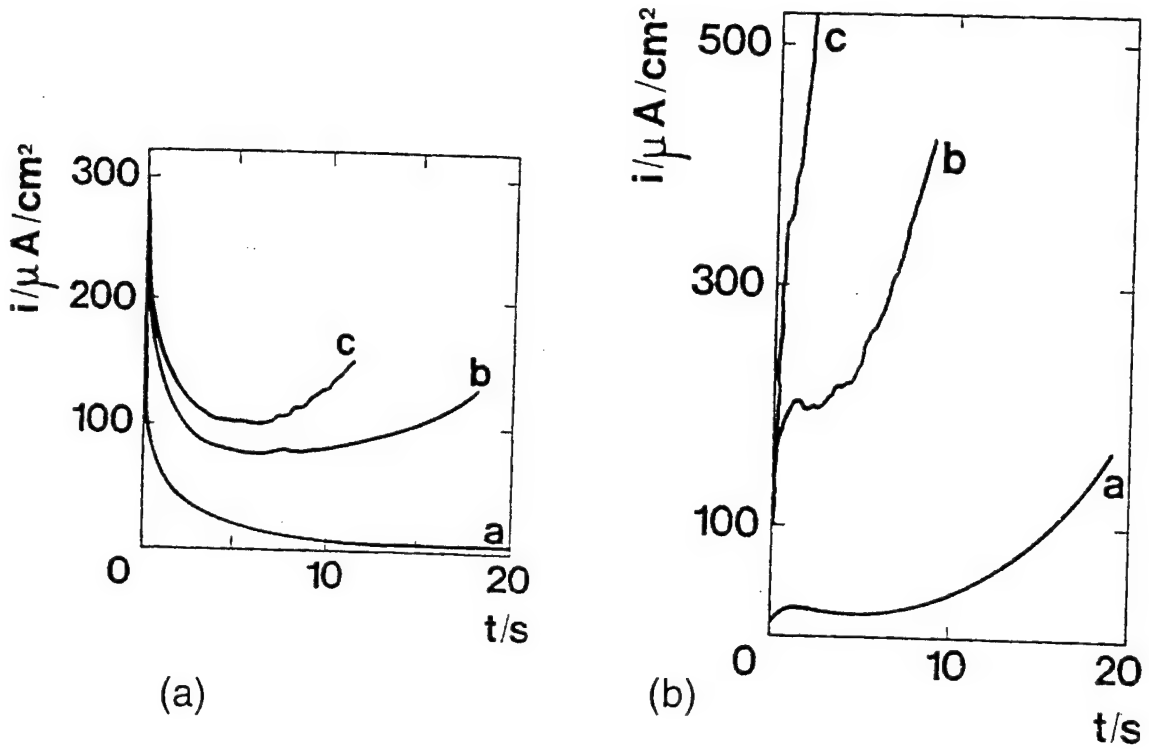


FIGURE 75 - Current transients at constant applied potential for 2024 aluminum in (a) 0.01 M NaCl and (b) 0.01 M NaCl + 0.05 M citric acid; applied potentials: -0.55 V vs SCE (curve a), -.50 V (curve b), and -0.48 V (curve c).[24]

which E_{pit} is determined to be the potential (E) at which the anodic current increases after application of the potential step, e.g., $E > E_{pit}$. When the experiment is performed under computer control, the value of E_{test}, magnitude of each and time at each potential step, and number of data points per second might have to be entered. The experiment will then be performed as programmed and the current-time plot will be displayed on the computer screen. After reformatting data, a printout of the current vs time curve can be obtained.

Videla determined E_{pit} = -0.55 V vs SCE in the potentiostatic mode in a study of the effect of fungal growth on the electrochemical behavior of aluminum.[24] Current transients at applied potentials were compared for 2024 aluminum in 0.01 M NaCl and in 0.01 M NaCl + 0.05 M citric acid (pH = 2.5), a fungal metabolite. At potentials more positive than -0.50 V, the current increased continuously after an induction time in NaCl (Figure 75a) and E_{pit} was determined as -0.50 V. Induction times were shorter in the acidified solution, and larger currents were recorded at the same E_{appl} (Figure 75b). In this case, E_{pit} was determined as -0.55 V. The author concluded that *Cladosporium resinae* plays an active role in the electrochemical behavior of 2024 aluminum. Since the decrease of E_{pit} was larger in fungal cultures of *Cladosporium resinae* than that observed in citric acid solutions at similar pH and Cl^- levels (Figure 75 b), it was assumed that other fungal metabolites participate in the pitting process.

Steady-State Current vs E_{appl}

The procedure described above is the same when a series of potential steps are applied in a potentiostatic test. Start the anodic polarization test by applying the first potential 10 mV to 50 mV more positive than E_{corr}. After the current (recorded continuously throughout the test on a strip-chart recorder) has reached a steady-state value or after a predetermined time, E_{appl} is increased to the next value and the current recorded. As increasing values of E_{appl} are used, it will become necessary to switch to less sensitive current scales. After

the current at the final E_{appl} value has been recorded, the polarization curve (E_{appl} vs log i) can be plotted using final values of current at each potential and converting it to current density. Figure 76 shows the typical standard potentiostatic anodic polarization plot for type 430 stainless steel (UNS S43000) in 1.0 N sulfuric acid (H_2SO_4) at 30°C. Polarization curves performed according to ASTM G5 ("Standard Reference Test Method for Making Potentiostatic and Potentiodynamic Anodic Polarization Measurements") with the standard ASTM sample will fall within the scatter band shown in Figure 76 if the instrumentation is properly calibrated and testing is performed correctly.

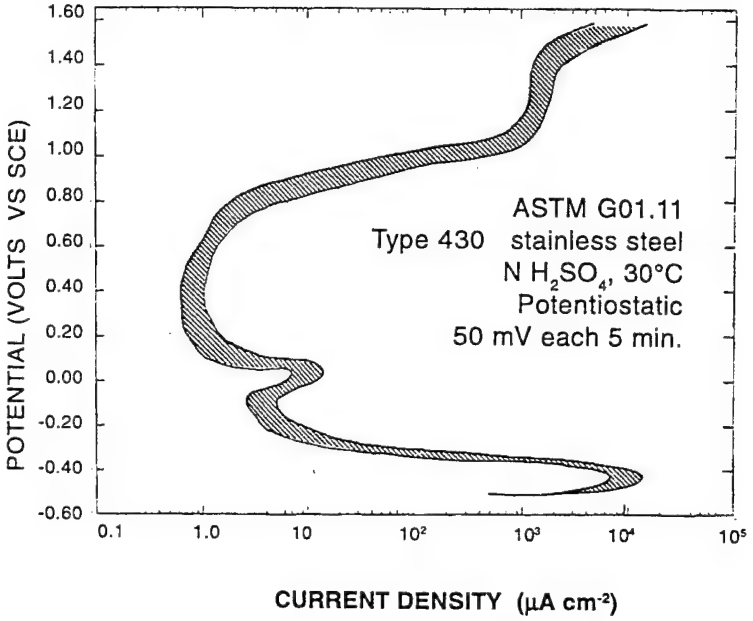


FIGURE 76 - Standard potentiostatic anodic polarization curve for stainless steel alloy 430 in 1.0 N H_2SO_4, 30°C (ASTM G5).

Potentiodynamic Techniques

Potentiodynamic polarization can be obtained as a single sweep, i.e. E_{appl} is increased at a constant scan rate (dE_{appl}/dt) between a starting potential (E_s), often E_{corr}, and a final potential (E_f). Sometimes the scan is reversed when E_f, or a preset maximum current density (i_{max}), is reached and stopped when the initial E_{corr} or a new value of E_{corr} is reached. In general, less distortion of the true polarization curve occurs at lower scan rates. In ASTM G5-94, a scan rate of 600 mV h^{-1} is recommended. Figure 77 shows the effects of varying scan rates on the shape of the anodic polarization curves for type 430 stainless steel (UNS 43000) in 1.0 N H_2SO_4. Scan rates were increased from curve 1 to curve 4.

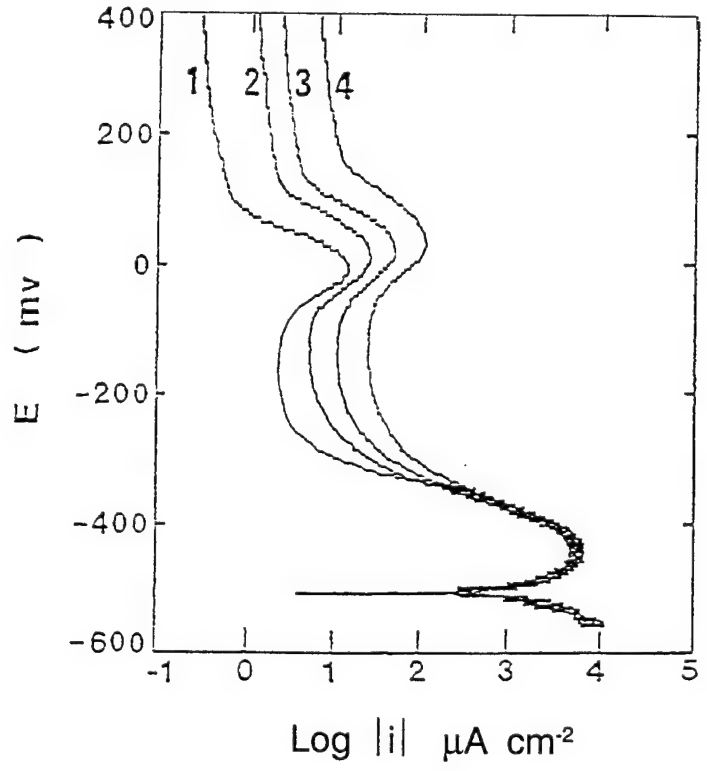


FIGURE 77 - Effect of scan rate on anodic polarization curve for SS alloy 430 in 1.0 N H_2SO_4, 30°C.[25]

Single Sweeps

Anodic and cathodic polarization curves usually are determined at a constant scan rate in a single sweep between two preset potentials E_s and E_f. ASTM G3 ("Standard Practice for Conventions Applicable to Electrochemical Measurements in Corrosion Testing") gives examples of polarization curves determined in this manner. Figure 78 shows the typical standard potentiodynamic anodic polarization plot for type 430 stainless steel in 1.0 N H_2SO_4 at 30°C. Anodic potentiodynamic polarization curves performed according to ASTM G5 with the standard ASTM sample will fall within the scatter band shown in Figure 78 if the test is performed correctly. Slightly different curves have been obtained with a new lot of type 430 stainless steel samples introduced in 1992. Deviations from standard plots point to deficiencies in the experimental technique or problems with the test cell or the equipment, and/or the operator.

Pitting Scans

Pitting scans (Figure 79) are carried out to determine E_{pit} and the protection potential (E_{prot}). ASTM G61-86 ("Standard Test Method for Conducting Potentiodynamic Polarization Measurements for Localized Corrosion Susceptibility of Iron-, Nickel-, or Cobalt-Based Alloys") gives a procedure for conducting cyclic potentiodynamic polarization measurements to determine

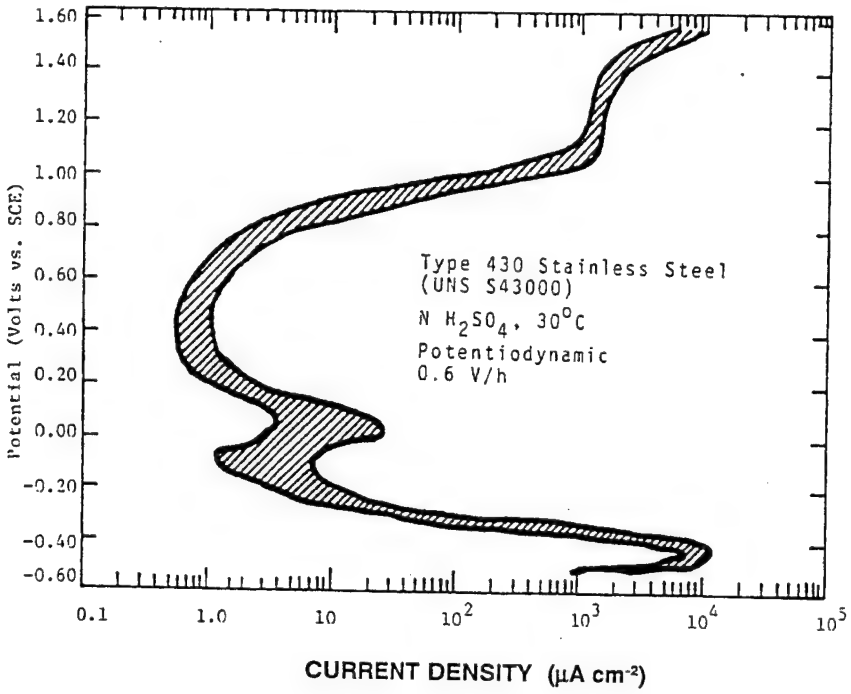


FIGURE 78 - Standard potentiostatic anodic polarization curve for stainless steel alloy 430 in 1.0 N H_2SO_4, 30°C (ASTM G5).

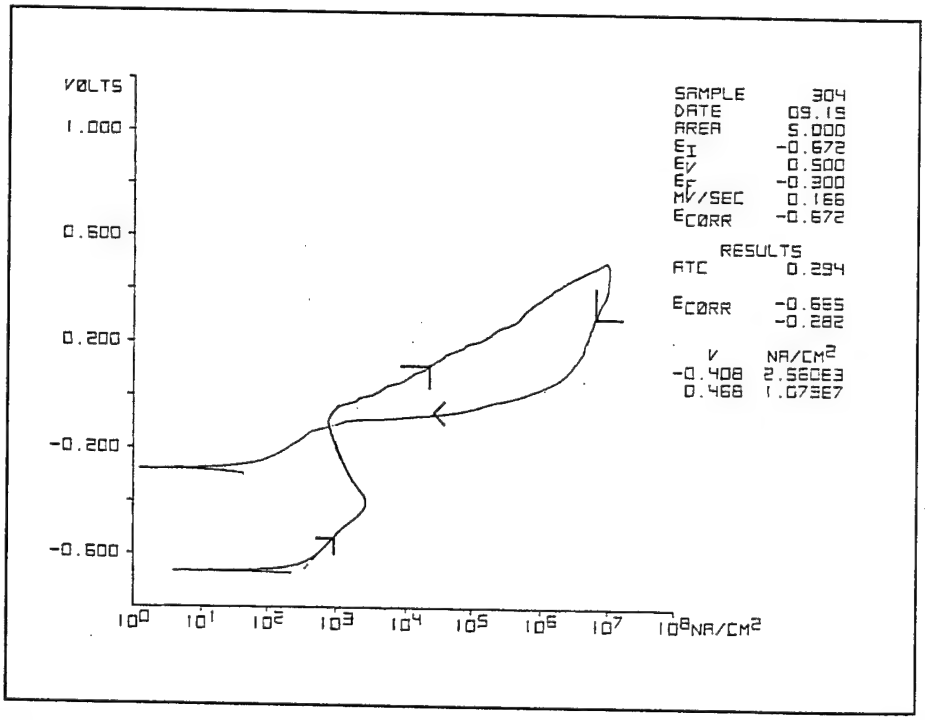


FIGURE 79 - Typical pitting scan plot. (Courtesy of EG & G Princeton Applied Research, Princeton, NJ)

relative susceptibility to localized corrosion in the form of pitting or crevice corrosion. An indication of susceptibility to localized corrosion is given by E_{pit}, the potential at which the anodic current increases rapidly from the low values in the passive region. The more noble E_{pit} is with respect to E_{corr}, the less susceptible the alloy is to initiation of localized corrosion. Cyclic potentiodynamic polarization curves for 304 stainless steel (UNS S30400) in a sodium chloride solution have a value of E_{pit} close to 0 mV vs SCE, indicating greater susceptibility to pitting than alloy C-276, which is immune to localized corrosion as indicated by the lack of hysteresis in the reverse scan (Figure 80). The area under the curve for the forward and reverse scan for a susceptible alloy such as 304 stainless steel is a qualitative measure of corrosion damage due to pit growth.

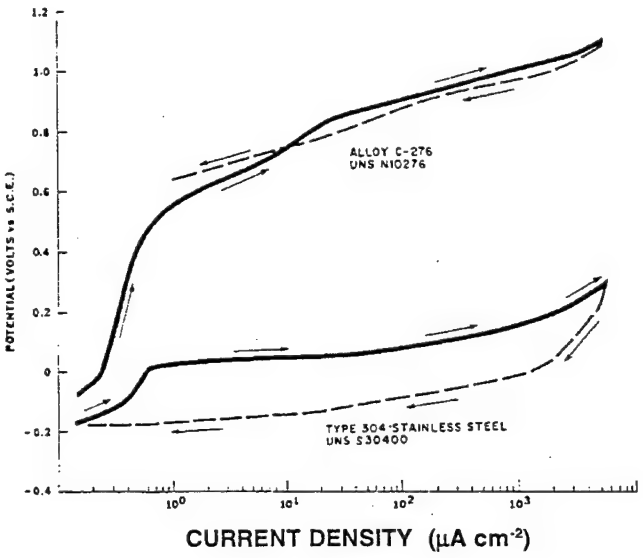


FIGURE 80 - Cyclic potentiodynamic polarization curves for stainless steel alloy C-276 and 304 in 3.56 w/o NaCl (ASTM G61).

Numerous investigators have used polarization curves to determine effects of microorganisms on electrochemical properties of metal surfaces and resulting corrosion behavior. In most studies, comparisons have been made between polarization curves recorded in sterile and inoculated media. Most authors suggest that microorganisms increase the rates of anodic and/or cathodic reactions. However, it also has been suggested that these rates can be reduced if corrosion-inhibiting compounds are produced by microorganisms.

Deshmukh et al. discussed the influence of sulfide pollutants on a naval brass exposed to polluted seawater based on potentiodynamic polarization curves. The shape of the anodic polarization curve was drastically changed in the presence of sulfides or SRB (Figure 81).[26] E_{corr} became substantially more negative. An active-passive transition and hysteresis in the reverse scan were observed.

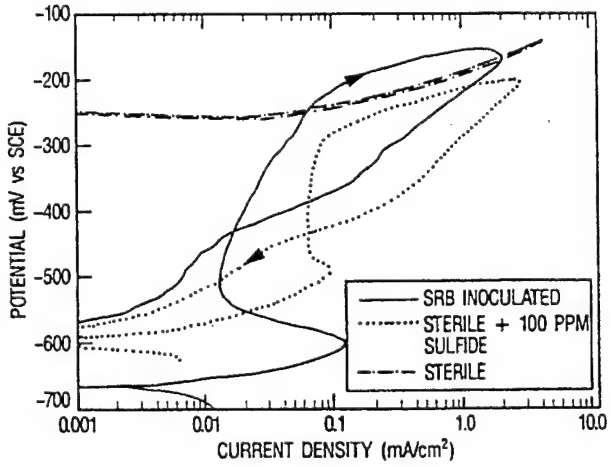


FIGURE 81- Potentiodynamic polarization curves for naval brass in seawater media.[26]

Polarization techniques also have been used to determine the mechanism by which microorganisms induce pitting or crevice corrosion. In most cases, E_{pit} has been determined in the presence and absence of bacteria. E_{pit} indicates tendency for pitting, but does not provide information related to the rate at which pits propagate. The extent of pitting cannot be predicted from E_{pit} data alone.

Salvarezza et al. used fungi isolates from jet fuel storage tank sludge to determine the influence of microorganisms on corrosion of aluminum alloys in fuel-water systems.[27] The authors concluded that E_{pit} could be used to determine the importance of each biological species in the corrosive attack of the aluminum alloys. Rosales and deSchiapparelli determined the influence of microbial contaminants in aircraft fuel, alloying elements and surface heterogeneities on nucleation and propagation of pitting of 7075 aluminum, concluding that electrochemical tests reproduced corrosion problems observed in service conditions.[28]

Ringas and Robinson performed electrochemical tests on several stainless steels and mild steel in cultures of SRB.[29] In all cases, the pitting resistance was lower in cultures of SRB.

Figure 82 shows potentiodynamic polarization curves with a reversed scan for stainless steel type 316 (UNS S31600) in a sterile medium and in a SRB culture. The alloy was passive and displayed a large passive range in the sterile medium. The reverse scan showed no hysteresis, indicating that active pits were not formed. The shape of the anodic polarization curve was very different in the bacterial culture. E_{corr} was more active, an active-passive transition was observed, and the passive current density was higher, suggesting that the passive film formed in the bacterial culture was less protective.

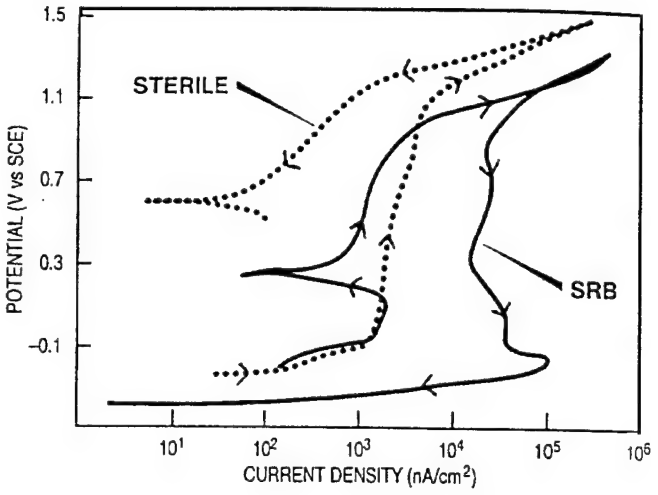


FIGURE 82 - Pitting scans for 316 stainless steel in sterile medium and in SRB culture.[29]

Polarization Curves Obtained in the Vicinity of E_{corr}

Polarization curves obtained in the vicinity of E_{corr} can be analyzed by fitting experimental data to the equation relating potential (E) and current density (i):

$$i = i_{corr}[\exp\{2.3\ (E - E_{corr})/b_a\} - \exp\{2.3\ (E_{corr} - E)/b_c\}]. \qquad (10)$$

Polarization curves usually are obtained in the potentiodynamic mode. Results of the fit using software programs consist of experimental values of i_{corr}, b_a, and b_c. Most potentiostat manufacturers supply software for this procedure. Since only a small polarization range ($E - E_{corr}$) is applied (usually ± 30 mV or less) to obtain corrosion rates and Tafel slopes in the non-Tafel region, this approach is non-destructive and can be applied repeatedly without changing surface properties. The experimental approach is similar to that used to determine R_p. However, kinetic data are obtained directly from polarization curves.

Figure 83 gives a comparison of an experimental polarization curve recorded in the vicinity of E_{corr} using a rotating cylindrical electrode (RCE) at a rotation speed r = 1,600 rpm and a fit of these data to Equation 10 for 99copper after exposure to natural seawater for seven days. Excellent agreement between these two curves was observed. E_{corr} and the fit parameters b_a, b_c, and i_{corr} have been plotted vs $r^{0.7}$ (rotation dependence for a

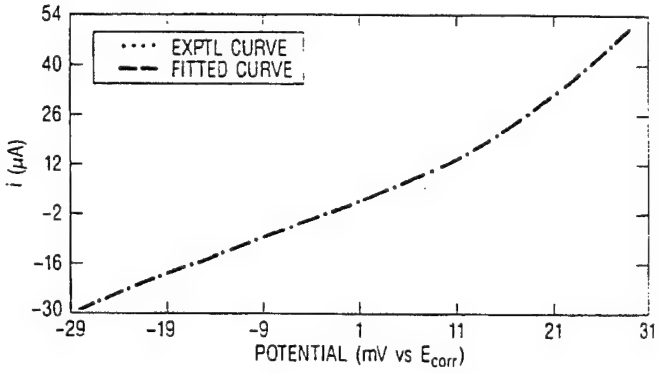


FIGURE 83 - Comparison of experimental and fitted polarization curves for 99copper after exposure to natural seawater for seven days; RCE, 1,600 rpm.[30]

RCE) for 99copper and 70copper:30nickel in Figure 84 after exposure to natural seawater for 7 and 21 days. These results have been interpreted in terms of the anodic reaction being under mixed charge transfer and mass transport control. It is not possible to obtain the kinetic data as a function of r (Figure 84) with traditional polarization measurements in a wide potential range since the surface would suffer irreversible changes during a measurement at the first rotation speed.

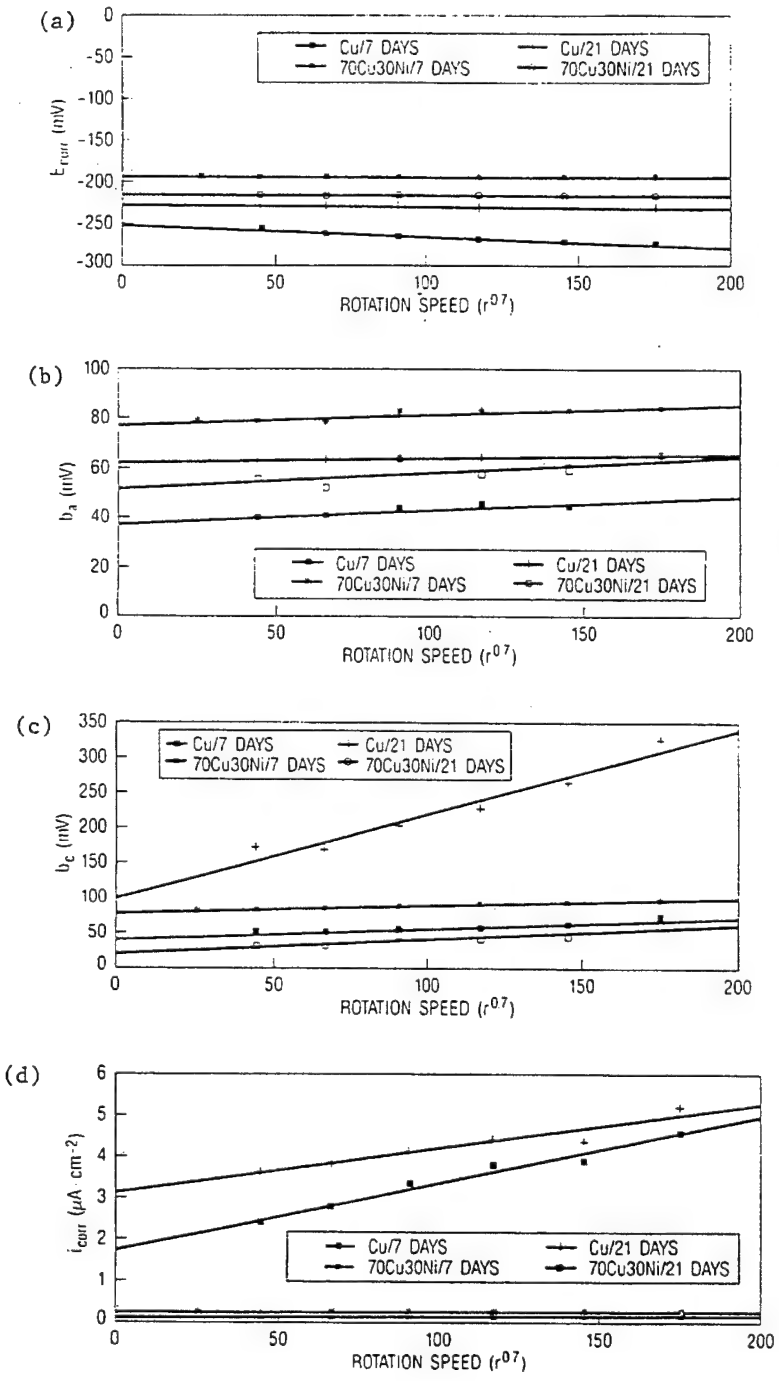


FIGURE 84 - Dependance of (a) E_{corr}, (b) b_a, (c) b_c, and (d) I_{corr} on rotation speed of RCE for 99copper (Cu) and 70copper:30nickel after exposure to natural seawater for 7 and 21 days.[30]

Polarization Resistance Measurements

Polarization resistance (R_p) defined as

$$R_p = (dE/di)_{Ecorr} \qquad (11)$$

is determined as the slope of a polarization curve at E_{corr} where i = 0. Usually, potentiodynamic polarization curves collected in close vicinity of E_{corr} are used for this purpose. ASTM G 59-91, "Standard Practice for Conducting Potentiodynamic Polarization Resistance Measurements," deals with experimental procedures for determining R_p according to Equation 11. This method describes an experimental procedure for Rp resistance measurements that allows an investigator to calibrate equipment and test methods.

Figure 85 shows standard polarization curves for type 430 stainless steel (UNS S43000) in 1.0 N H_2SO_4, deaerated with hydrogen, at 30°C. Experimental polarization curves must fall within curves 2 and 3; otherwise, experimental problems have occurred.

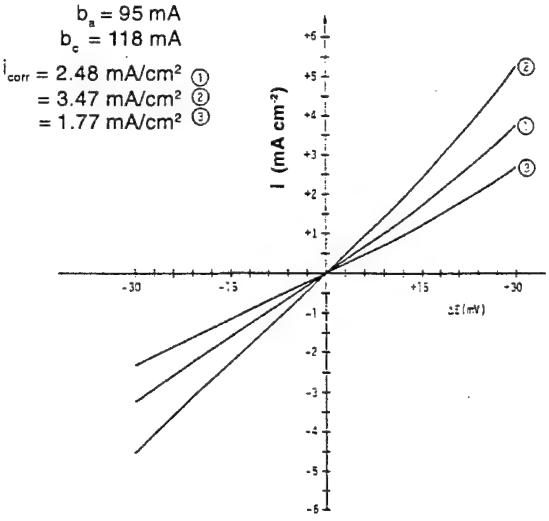


FIGURE 85 - Standard potentiodynamic polarization curves in the vicinity of E_{corr} for 430 stainless steel in 1.0 N H_2SO_4, 30°C (ASTM G59).

Table 6.1 gives results of the analysis of experimental data obtained in round robin testing. The mean values (M) of R_p, b_a, b_c, and i_{corr} are given with their standard deviations (SD) and standard errors (SE). The analysis was carried out as discussed in relation to Equation 10. Curves 2 and 3 were constructed for M +/ - 2 SD.

Applications of the R_p techniques as a non-destructive method for monitoring corrosion rates have been discussed in detail in a review article.[31] The main advantage of the R_p technique over periodic weight loss measurements is the possibility of continuously monitoring instantaneous corrosion rates of a metal exposed to a corrosive environment. The technique is suitable for detection of changes due to the presence of bacteria, inhibitors, biocides, sunlight, changes in flow rate and other environmental conditions.

R_p can be converted to i_{corr} using the Stern-Geary equation:[21,31]

$$i_{corr} = B/R_p , \qquad (12)$$

where B is a parameter calculated using the Tafel slopes:

$$B = b_a b_c/2.3(b_a + b_c) . \qquad (13)$$

To obtain quantitative values of i_{corr} from experimental R_p data, B has to be known for the same experimental conditions. For qualitative estimates, a constant value of B between 13 and 26 mV often is used. Corrosion rates can be obtained from i_{corr} using Faraday's Law.[23]

In determining R_p values from polarization curves, scan rate and ohmic drop effects have to be considered. Experimental polarization curves can change with increasing scan rate. In general, R_p will be underestimated (and corrosion rates overestimated) when scan rates are too high. R_p will be overestimated (and corrosion rates underestimated) when the uncompensated resistance (R_s) is not considered since the experimental value of R_p ($R_{p,exp}$) contains a contribution from R_s:

$$R_{p,exp} = R_p + R_s . \qquad (14)$$

The contribution from R_s can be eliminated during the measurement by using positive feedback or interrupter techniques or by subtracting R_s from $R_{p,exp}$ as calculated from analysis of the polarization curve.[31] R_s can be determined by applying a constant current pulse I_{appl} and observing the potential response on an oscilloscope (Figure 86). The initial sharp rise of potential $E = I_{appl}R_s$ due to ohmic drop is followed by a slower increase due to double layer charging. Thus, $R_s = E/I_{appl}$.

The error $= R_s/R_p$ due to the uncompensated resistance can have similar values for a system with high corrosion rates in a solution of high conductivity and for a system with low corrosion rates in a solution of low conductivity. Therefore, ohmic drop effects can be important in all systems.

TABLE 6.1
POLARIZATION DATA FOR STAINLESS STEEL TYPE 430, 1 N H_2SO_4, H_2, 30°C (ASTM G59)

	M	SD	SE
R_p (ohm · cm²)	9.19	1.54	0.54
b_a (mV)	95	18	6
$b_{c\,(mV)}$	118	16	6
B (mV)	22.8	3.6	1.3
I_{corr} (mA/cm²)	2.49	0.19	0.07

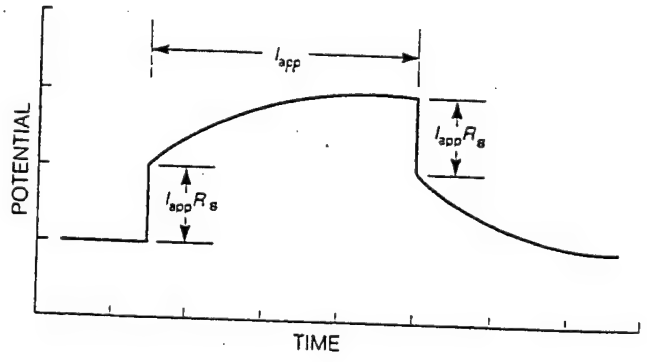


FIGURE 86 - Potential pulse method for determination of the uncompensated resistance R_s.[22]

Linear Polarization

The linear polarization technique is a variation of the polarization resistance technique and forms the basis of commercial corrosion rate monitors. In this approach, it is assumed that polarization curves are linear in the vicinity of E_{corr} (E_{corr} ± 10 mV). In this case, R'_p is defined as:

$$R'_p = \Delta E/i \,, \qquad (15)$$

where $\Delta E = E - E_{corr}$ and i is the current measured at the applied potential E. Since ΔE is constant, i_{corr} is proportional to the measured current density.[31]

$$i_{corr} = k\, i \,, \qquad (16)$$

where k is treated as a system constant.

In commercial instruments, positive and negative values of ΔE are applied and the average value of i used to determine the corrosion rate. The assumption that the polarization curve is linear with ΔE has been discussed and the limitation of the technique has to be considered in each application.[31] Several commercial devices are available, and automatic ohmic drop compensation is available in some instruments.

Mansfeld et al. used linear polarization techniques to determine R_p for mild steel sensors embedded in concrete.[32] Concrete samples were exposed to a sewer environment in Los Angeles County for about nine months. One sample was periodically flushed with sewage in an attempt to remove the acidic environment produced by sulfur-oxidizing bacteria, and another sample was used as a control. A data logging system was used to collect R_p data at 10 min intervals simultaneously for two corrosion sensors and two pH electrodes placed on concrete surfaces. Figure 87 shows the cumulative corrosion loss ΣINT, where INT was obtained by integration of the $1/R_p$- time curve (area under the $1/R_p$- time curve) as:

$$INT = \int \frac{dt}{R_p} \qquad (17)$$

The data in Figure 87 qualitatively represent the time dependence of the mass loss of the steel electrodes.

No attempts were made to convert R_p data into corrosion rates since the B values (Equations 12 and 13) were not known for the system and the purpose of the test was to evaluate whether the concept of removing the acidic environment by periodic flushing with sewage was feasible. A qualitative measure of corrosion rate can be obtained from the slope of curves in Figure 87. The integrated corrosion loss (INT) is given in Figure 87 in units of s/ohm. Corrosion rates started to increase for both samples after about 60 days when apparently enough electrolyte had reached the steel surfaces to make corrosion possible. A significant decrease of corrosion rates occurred after about 111 days when flushing was started (Figure 87b). Total corrosion loss as determined from the integrated R_p data was less for the control than for the flushed sample.

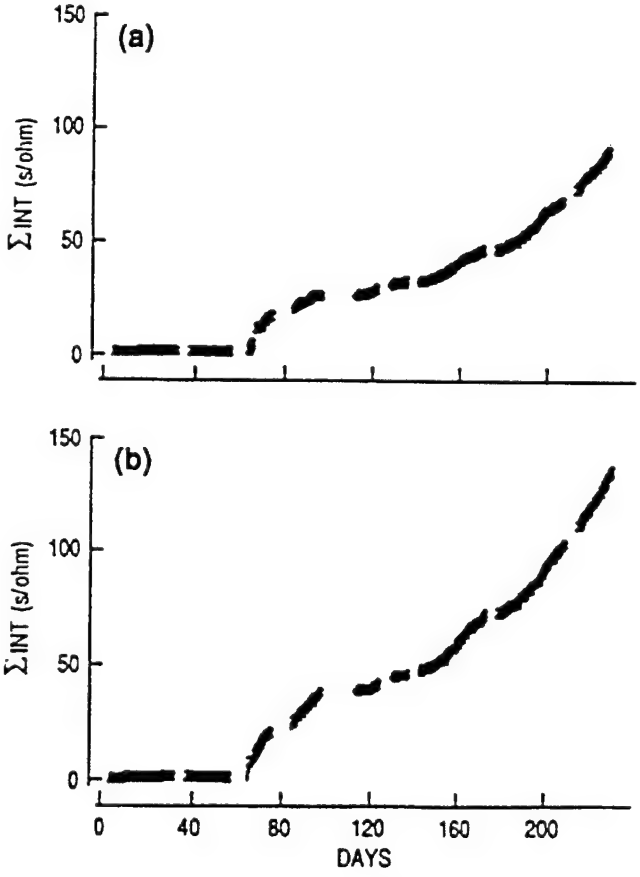


FIGURE 87 - Cumulative corrosion loss ΣINT for mild steel sensors embedded in concrete and exposed to a sewer bypass. (a) Control sample and (b) flushed sample.[32]

Galvanostatic and Galvanodynamic Techniques

Galvanostatic and galvanodynamic techniques are used less commonly than the potentiostatic and potentiodynamic techniques described earlier. In galvanostatic techniques, a constant current is applied and potential is monitored as a function of time. It is possible to record an entire polarization curve in the galvanostatic mode. A limitation of this approach for passive metals is that the passive region cannot be investigated.[21] ASTM G 100, "Standard Test Method for Conducting Cyclic Galvanostaircase Polarization," describes a method for determining E_{pit} for aluminum alloys using a variation of the galvanostatic technique.

Open-Circuit Potential Measurements

Measurement of E_{corr} is the easiest electrochemical test to perform, but it provides the least amount of mechanistic information. Measurement of E_{corr} requires a stable reference electrode, a high impedance voltmeter, and, in most cases, a suitable recording device. E_{corr} also can be determined by observing the digital display on the potentiostat when i = 0, monitored by recording the potential output value from the potentiostat on a strip chart recorder as a function of exposure time, or by printing potential vs time curves stored on a computer disk.

The time dependence of E_{corr} has been monitored by many groups investigating effects of biofilm formation on localized corrosion of stainless steels in natural seawater. Most published data show a rapid ennoblement of E_{corr} (i.e., a shift to more positive values) during the first days of exposure as seen in Figure 88 for several stainless steels in flowing (0.5 m s^{-1}) natural seawater. E_{corr} changed from -200 to -250 mV (SCE) at the beginning of the test to -50 mV for two alloys and to more than +50 mV for the remaining four alloys after 28 days.[33]

It is important not to overinterpret E_{corr} measurements. Based on information obtained with E_{corr} alone, it is not possible to determine whether corrosion rates have increased or decreased. Some authors have interpreted shifts of E_{corr} in the negative direction as a reduction of the rate of cathodic reaction or an increase of the rate of the anodic reactions. These are the two simplest possibilities. However, without additional data, no valid conclusions concerning the effect of bacteria on electrochemical corrosion reactions can be drawn. The phenomenon of ennoblement observed by many investigators has been explained by the experimentally determined increase of the rate of the cathodic reaction due to biofilm formation. However, no satisfactory mechanistic explanation has been provided to account for this phenomenon.

The reduction-oxidation (redox) potential measured on an inert electrode such as platinum can be used as an indicator of the oxidation power of the electrolyte. If suitable calibration is provided, it might also be used as an indicator of electrolyte corrosivity. Measurements of redox potentials have been used to monitor changes in corrosive properties of solutions as a result of bacterial metabolism. Potential measurements of both a prepassivated platinum electrode (passivated by immersion in 25% HNO_3 + 75% H_2O at 50°C for 10 min) and the material of interest has been used to follow biofilm development and determine its effects on the corrosion behavior of structural materials.

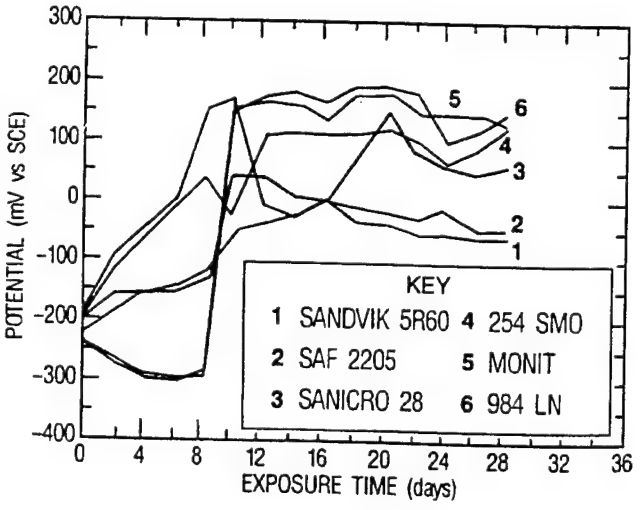
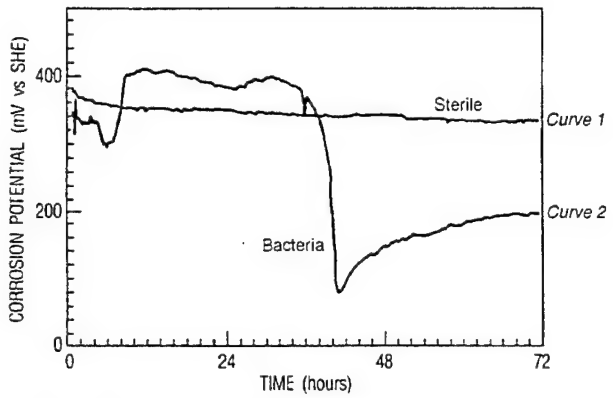


FIGURE 88 - Open circuit potential as function of time for six stainless steels exposed to flowing natural seawater.[33]



SHE - Standard hydrogen electrode

FIGURE 89 - Open circuit potential vs time for 304L stainless steel in sterile medium (curve 1) and in medium with bacteria (curve 2).[34]

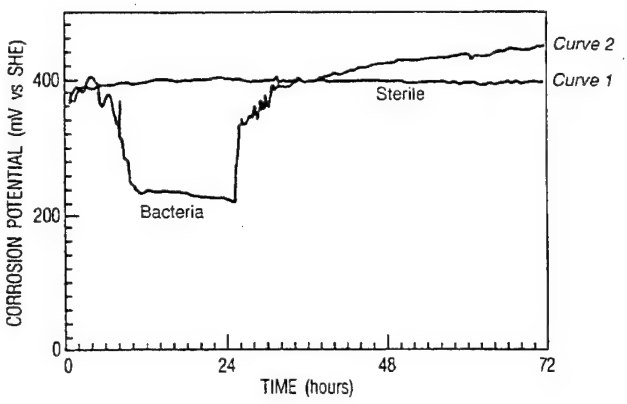


FIGURE 90 - Open circuit potential vs time for platinum in sterile medium (curve 1) and in medium with bacteria (curve 2).[34]

Figure 89 shows the time dependence of E_{corr} for stainless steel type 304L (UNS S30403) in solutions with and without bacteria, while Figure 90 shows corresponding data for prepassivated platinum, the role of which was to indicate changes in oxygen concentration at the platinum/biofilm interface, assumed to be the same as those on stainless steel.[34] Since the potential of platinum changes in the positive direction when the oxygen concentration is increased or pH is decreased, a pH indicator was added to the solution to determine pH changes independently. E_{corr} for 304L and platinum in the sterile medium remained essentially constant over the 72 h test period (Figures 89 and 90) while in the solution containing bacteria significant potential changes were observed for 304L accompanied by a decrease in solution pH. The potential of platinum also changed with time in the bacterial solution, but in the opposite direction (Figure 90). The authors concluded that potential changes corresponded to bacterial activity.

Galvanic Current Measurements

Short-circuit currents between dissimilar metals or metals exposed to electrolytes of different composition are measured by means of a zero resistance ammeter (ZRA). The ZRA is based on operational amplifiers (Figure 91) and applies 0 mV potential difference between the two electrodes in the galvanic couple. The potential of the couple should be measured vs a reference electrode to obtain additional information related to corrosion mechanisms. A potentiostat can be used as a ZRA by setting the applied potential to 0 mV. The working electrode lead is connected to one electrode while the counter electrode lead and the reference electrode lead are connected to the other electrode. Galvanic current is monitored as current output of the potentiostat.

When using a ZRA based on operational amplifiers, one electrode is connected to the grounded input (Figure 91). The current (I_g) needed to maintain 0 mV between two galvanically coupled electrodes is monitored as the output voltage $V_o = I_g R_m$, where R_m is the

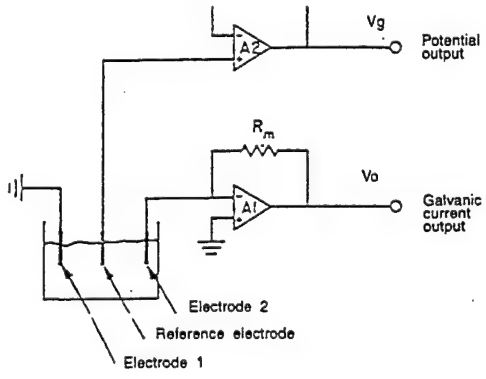


FIGURE 91 - Instrumental arrangement for continuous measurement of galvanic current at zero impedance.[35]

resistance of the measuring resistor. R_m can be varied to maintain V_o in a convenient range, i.e. 0-1 V. The voltage of the couple (V_g) is measured with the voltage follower (A2 in Figure 91) which determines the potential of the reference electrode vs ground. Both V_o and V_g can be recorded. The advantage of the design in Figure 91 is low cost and capability to monitor multiple test cells simultaneously. Operational amplifiers also can be used to construct inexpensive and versatile potentiostats.

A dual-cell corrosion measuring device that uses a ZRA has been developed specifically to evaluate vulnerability of metals to media containing microorganisms and to determine corrosivity of metal/microbe combinations. The dual-cell or split-cell technique allows continuous monitoring of a metal or alloy covered by a biofilm. Dual-cell devices are not commercially available, but can be easily constructed (Figure 92a). Basic features of the device include a support for a 0.1 µm cellulose acetate/cellulose nitrate filter disk that provides electrolytic continuity and microbiological isolation between two identical half cells. Nominally identical test electrodes are placed in electrolytes that differ only by the presence of microorganisms in one half cell and are connected to a ZRA. The operating principle of the device is based on the fact that as long as uniform corrosion occurs at the same rate on electrodes in both compartments and E_{corr} is the same, there should be no potential difference between the compartments and current flow between them should be zero. When one compartment is chemically or biologically altered, a potential difference will develop and a current can be measured. Compartments are provided with ports for gas exchange and introduction of electrolyte solutions.

This is the experimental procedure for using the dual cell:

• Autoclave all glassware and filters (15 psi steam at about 121°C for a minimum of 20 min). Assemble the device.

• Prepare abiotic solution using a combination of pasteurization and filtration. Pasteurize natural water for 2 h at 70°C before filtration through a 0.1 µm cellulose acetate/cellulose nitrate filter. Add abiotic electrolyte to both half cells.

• Clean electrodes in acetone and dry in warm, sterile air. Assemble identical test electrodes in electrode holders and place one in each half cell. Test electrodes need to be of equal dimensions and surface finish. Caution should be taken to ensure that electrode holders do not leak and that there are no crevices.

• If a potentiostat is used as ZRA, the following connections have to be made (Figure 92b):

Connect working electrode lead to one test electrode, counter electrode and reference electrode leads to the other test electrode.

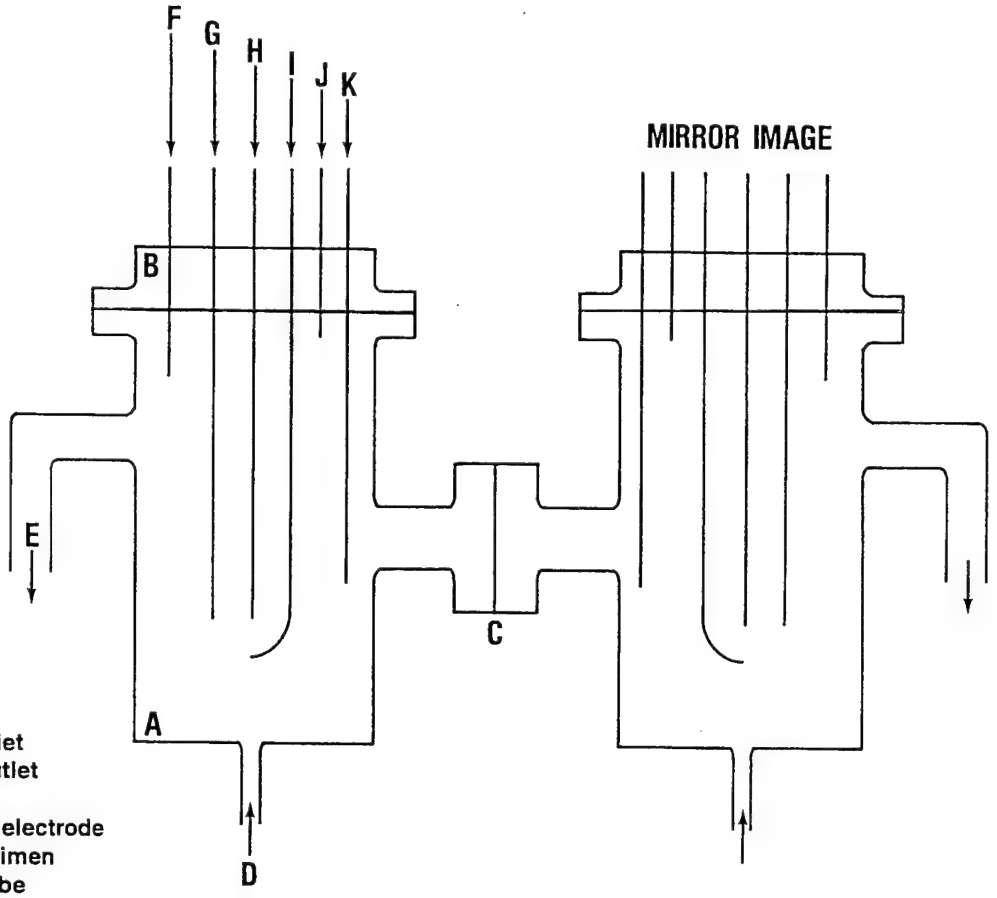
Set applied potential to 0 mV, adjust current scale, and measure galvanic current for several hours to make sure that the current is very low.

• Inoculate one half-cell.

• Record galvanic current with a strip chart recorder connected to the current output of the potentiostat or ZRA to provide a continuous record of the time dependence of the corrosion process. If appropriate software is used, data can be displayed on a computer screen during the test, stored on disk, and plotted after the test. Typical test times last from days to months.

• The potential of the galvanic couple may be monitored by connecting the reference electrode to one input of an external voltmeter and the test electrode to which the working electrode lead is connected to the low or grounded input. The output voltage may be connected to a second strip chart recorder (Figure 92b).

Galvanic current is a measure of the increase of the corrosion current of the anode due to the coupling to the cathode and does not allow calculation of the corrosion rate of either test electrode. The sign and magnitude of the galvanic current can be used to determine details of the corrosive action of the bacteria. However, care must be taken in determining the sign of the current since in principle either of the two test electrodes can be connected to the working electrode or the reference electrode + counter electrode leads. If in doubt, a quick check can be carried out by placing a piece of zinc and a piece of steel in tap water, connecting the zinc to the working electrode lead and the steel to the reference electrode + counter electrode lead. Current should be positive. If the sign of the current in a dual-cell experiment is positive and the test electrode in the bacteria-containing cell has been connected to the working electrode lead, it has become the anode (i.e., it has the more negative E_{corr}); and if the sign of the current is



A - Half cell
B - Cell cover
C - Filter
D - Medium inlet
E - Medium outlet
F - Utility port
G - Reference electrode
H - Metal specimen
I - Luggin probe
J - Gas outlet
K - Gas inlet

FIGURE 92(a) - Diagram of dual cell corrosion measuring device.

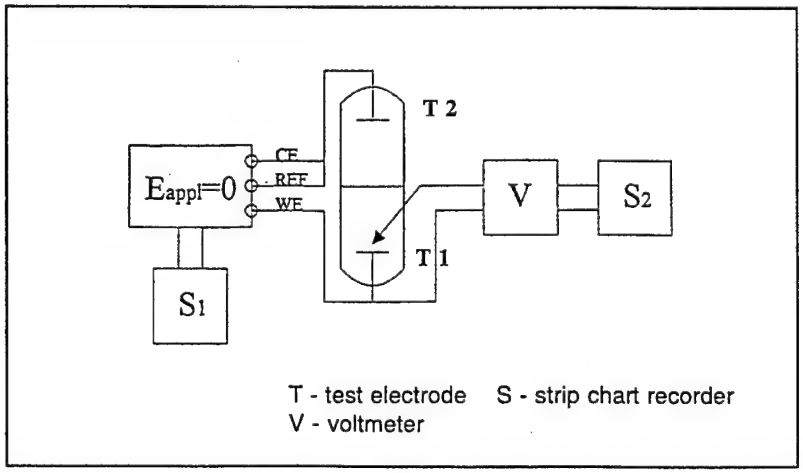


FIGURE 92(b) - Connections for galvanic current measurements when using potentiostat as ZRA.

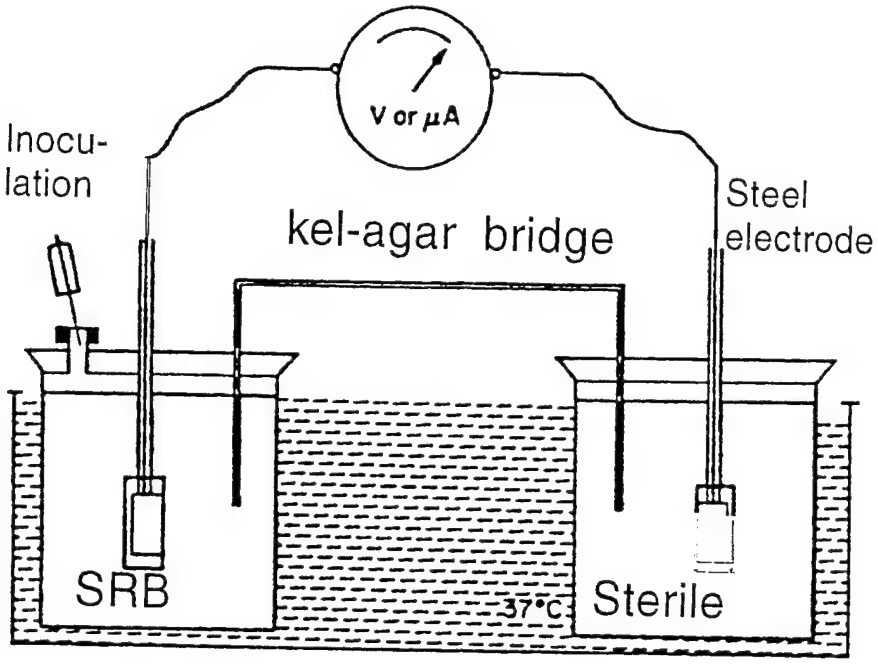


FIGURE 93(a) - Two cell microbiological battery used in SRB corrosion studies.[37]

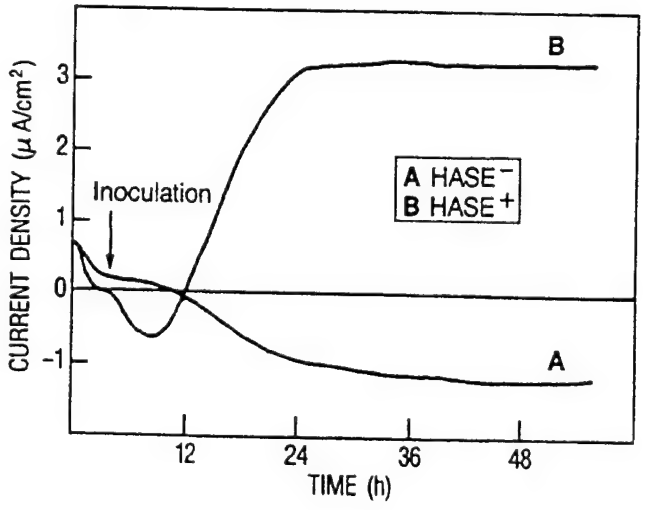


FIGURE 93(b) - Time dependance of the ZRA current in the presence of SRB Hase⁻ (curve A) and SRB Hase⁺ (curve B).[37]

negative, it has become the cathode. Simultaneous measurement of the galvanic current and the potential of the coupled test electrodes using a reference electrode allows one to determine changes in corrosion reaction kinetics caused by bacteria with exposure time. The magnitude of measured ZRA current depends on the area ratio of anode and cathode (A^a/A^c). If this ratio is not one, the measured galvanic current (I_g) has to be normalized to determine the galvanic current densities ($i_g^a = I_g/A^a$ and $i_g^c = I_g/A^c$), which will have different numerical values.

Daumas et al. studied a microbiological battery to determine the corrosion mechanism of a mild steel in the presence of hydrogenase-positive (Hase⁺) and hydrogenase-negative (Hase⁻) bacteria.[37] Current flowing between the test electrode in the sterile cell and the test electrode in the inoculated cell in the presence of Hase⁻ SRB (curve A) and in the presence of Hase⁺ SRB (curve B) was measured using the arrangement shown in Figure 93(a) and is presented in Figure 93(b) as a function of time. According to the authors, the difference between curves A and B shows the importance of the hydrogenase activity of SRB in the electrochemical process of corrosion. They concluded that oxidation of cathodic hydrogen was the dominant mechanism for Hase⁺ organisms and that FeS played a role in the corrosion reaction with Hase⁻ organisms.

Monitors

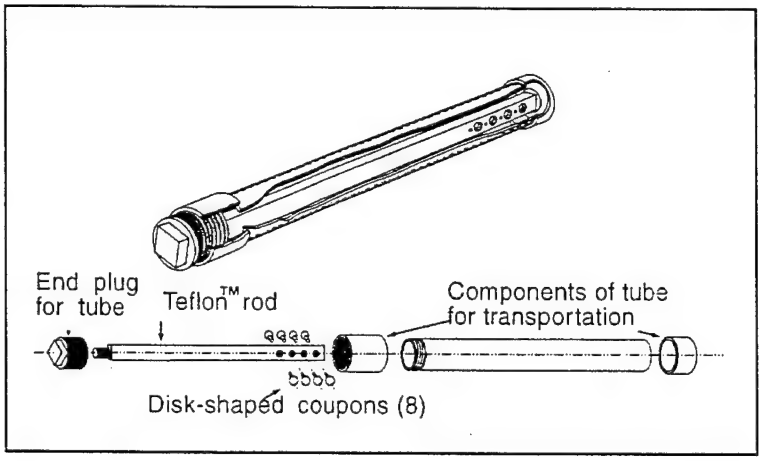


FIGURE 94(a) -Biofouling/corrosion monitoring system.[38]

Commercial multipurpose metal sample holders are available for MIC monitoring (Figures 94a-c). One sample holder consists of a rectangular section Teflon™ rod in which holes are drilled to support eight disk-shaped coupons, each with an exposed surface area of 0.5 cm^2. Four coupons are supported on one side of the rod while the other four are supported on the opposing side (Figure 94a). Similarly, the Robbins Device (94b) incorporates aseptically removable stubs to collect biofouling/corrosion information. Manufacturers provide design options, including stub and device

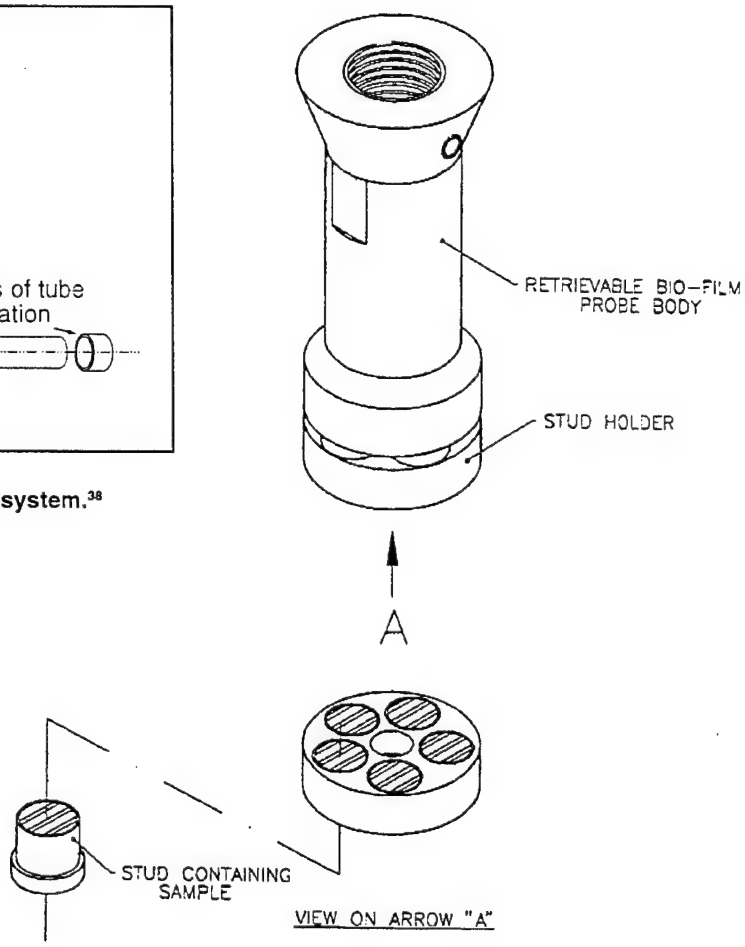


FIGURE 94(b) - Schematic of Robbins Device. (Courtesy of Caproco Ltd., Edmonton, Alberta, Canada.)

materials, sizes, and stub numbers. Continually updated designs allow similar devices to be mounted on spools for easy transport from one in-line or by-pass site to another (Figure 94c).

Sample holders can be supported in a conventional corrosion coupon rack and placed in a process sidestream of interest (Figure 95). In this way, eight different samples can be collected simultaneously or at different exposure periods. After exposure, entire assemblies or coupons can be removed for biofilm and corrosion assessment.

A typical monitoring scheme would include:

- sessile bacterial counts,
- biofilm observation by SEM,
- surface analysis of corrosion products and biofilms,
- optical microscopy and SEM evaluation of metal attack after removal of biological inorganic deposits,
- electrochemical corrosion measurements in the laboratory or the field to assess localized corrosion.

These studies are generally complemented with field and laboratory measurements, such as:

- water quality control,
- field corrosion monitoring,
- field redox potential measurements,
- other analytical data depending on particular characteristics of each system (dissolved oxygen, sulfides, etc.).

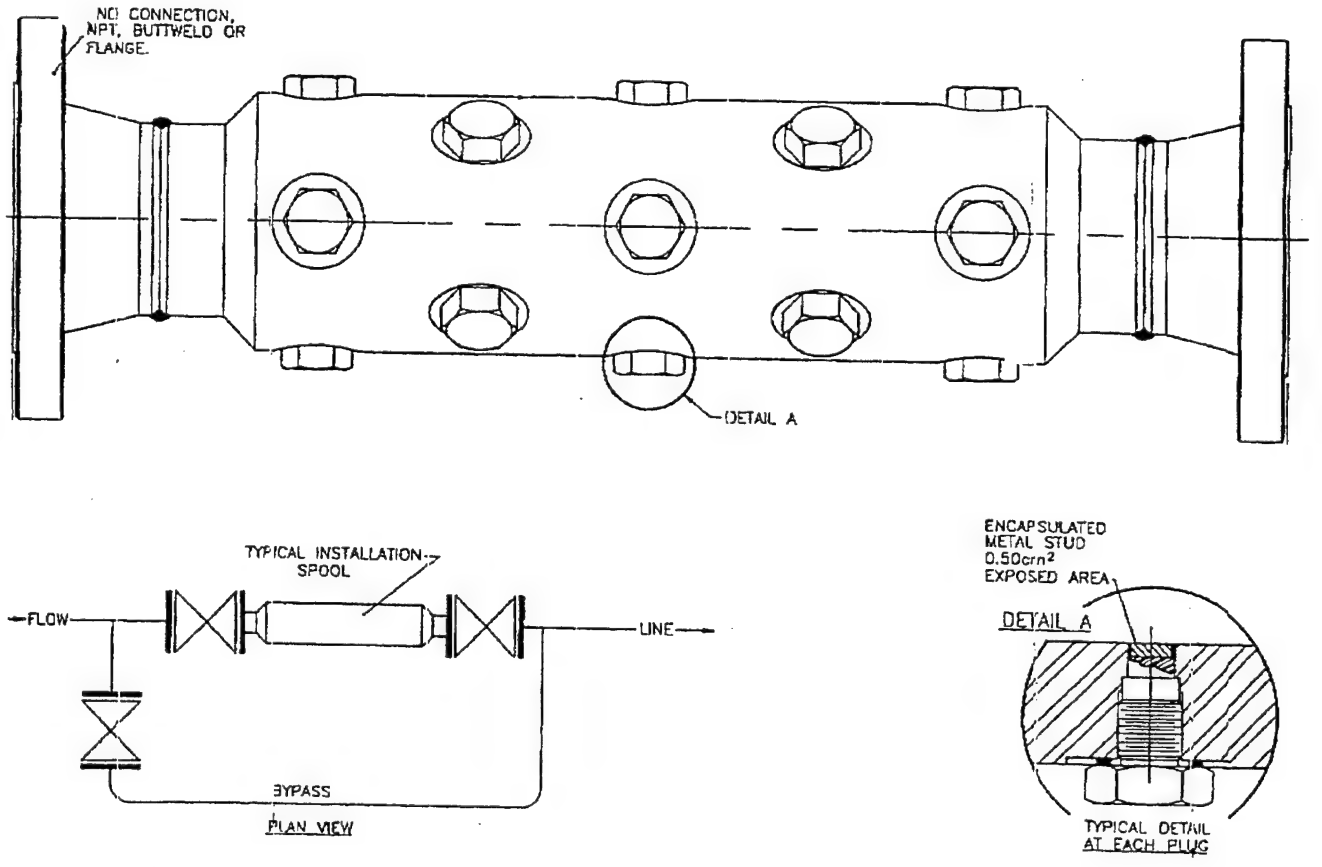


FIGURE 94(c) - Schematic of spool-mounted monitoring device (Courtesy Caproco, Ltd. Edmonton, Alberta, Canada.)

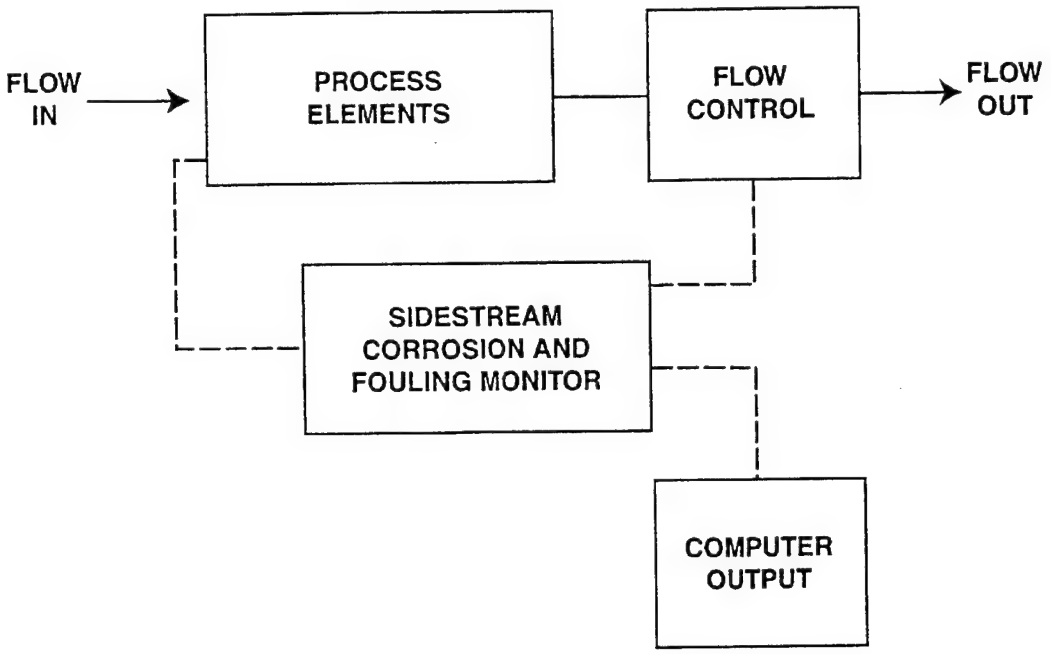


FIGURE 95 - Sidestream monitoring scheme.

CHAPTER 8

Recent Laboratory Developments

Innovations in microbiology, electrochemistry, and surface chemistry are being applied to MIC research in controlled laboratory situations. The following section describes some innovative methods and includes references for additional information. In most cases, developing techniques require sophisticated equipment and generally are not appropriate for routine testing.

Microbiological/Biochemical Techniques

Biofilm community structures can be analyzed using cluster analysis of the phospholipid fatty acid profiles (PLFA).[39] PLFA profiles for natural biofilms are more complex than profiles for laboratory biofilms. None of the laboratory profiles clustered closely with profiles from natural biofilms. In addition, the PLFA profiles for attached bacteria clustered separately from profiles of the same bacteria in the bulk phase, suggesting that either the community or the physiology of attached bacteria differ from that of bulk phase bacteria.

Direct molecular characterization of natural microbial populations can be accomplished with sequence analysis of 5S rRNA.[40] More recently, fluorescent dye-labeled oligonucleotide probes have been used for microscopic identification of single cells and characterization of mixed populations. Polymerase chain reaction amplification, comparative sequencing, and whole cell hybridization have been combined to selectively identify and visualize SRB in both established and developing multispecies biofilms.[41]

Techniques for analyzing microbial metabolic activity at localized sites are being developed. Franklin et al. incubated microbial biofilms with ^{14}C-metabolic precursors and autoradiographed the biofilms to localize biosynthetic activity on corroding metal surfaces.[42] The localized uptake of labeled compounds was related to localized electrochemical activities associated with corrosion reactions.

Electrochemical Techniques

Three non-destructive electrochemical techniques that can be applied in MIC studies are electrochemical impedance spectroscopy (EIS), electrochemical noise analysis (ENA), and biofilm monitoring probes.

EIS

Electrochemical impedance spectroscopy (EIS) data are recorded as a function of frequency of an applied AC signal at a fixed working point (E, i) of a polarization

curve. In corrosion studies, this working point is often E_{corr} ($E = E_{corr}$, $i = 0$). Usually, a very large frequency range has to be investigated to obtain the complete impedance spectrum. In most corrosion studies, this frequency range extends from 65 kHz, the upper limit of a commonly used frequency response analyzer (FRA), to 1 mHz. Impedance data are usually determined with a three-electrode system, although it also is possible to use a two-electrode system in which both electrodes are of the same material. A potentiostat is used to apply the potential at which data are to be collected.

The FRA is programmed to apply a series of sine waves of constant amplitude (small enough to remain in the linear potential region) and varying frequency. Impedance data are determined by the FRA at each frequency and stored in its memory. Since a very large number of data points has to be collected, displayed, and analyzed, it is essential to use adequate software for these purposes.

Information obtained with EIS differs from that determined with the other techniques previously described since the corrosion system is analyzed at a fixed potential (or current). Properties of the system at this potential can then be determined through analysis of the frequency dependence of the impedance. One of the advantages of EIS is that only very small signals are applied. In many cases, EIS data have shown that existing models for corrosion systems based on results of studies with direct current techniques were too simplistic.

Experimental impedance spectra can be displayed using linear coordinates in complex plane plots in which the negative value of the imaginary part of the impedance (-Z) is plotted vs the real part (Z). For a simple circuit consisting of a parallel combination of R_p and C with R_s in series (Figure 74), such a plot will result in a semicircle.

This approach neglects the fact that the impedance often changes over many orders of magnitude and that the frequency dependence of the impedance cannot be recognized in complex plane plots. The preferred format for display of EIS data is, therefore, the Bode plot, in which log |Z| and phase angle (Φ) are plotted vs the frequency (f) of the applied signal with |Z| being the modulus of the impedance.

ASTM G 106, "Standard Practice for Verification of Algorithm and Equipment for Electrochemical Impedance Measurements," describes an experimental procedure that may be used to check instrumentation and technique for collection and presentation of EIS data. Reference impedance plots in both complex plane and Bode format are included for a dummy cell and for stainless steel type 430 in a solution containing 0.005 M sulfuric acid (H_2SO_4) and 0.495 M sodium sulfate (Na_2SO_4).

Figure 96 shows Bode plots for 99copper as a function of exposure time to natural seawater over a 10-week period while Figure 97 shows data in artificial seawater. The most notable feature of the spectra recorded in natural seawater was the increase of impedance at high frequencies for longer exposure times (Figure 97), not observed in artificial seawater (Figure 96). Changes of |Z| with time are reflected in the frequency dependence of Φ, a sensitive indicator of changes in surface properties. Corrosion products formed in natural seawater were covered with a gelatinous biofilm of bacteria and crystalline deposits of copper, calcium, and phosphorous.

EIS data often have been used only to determine R_p, defined as the limit of the real part of the impedance for $f = 0$:

$$R_p = \lim_{f \to 0} \{ Re(Z) \} - R_s . \qquad (18)$$

In this case, all other important information contained in the impedance spectrum is ignored.

One requirement for obtaining valid impedance data is linearity, i.e., the applied signal must be small enough for the system to be linear. Electrochemical systems usually are nonlinear (Equation 10) except for small deviations from E_{corr}. Many studies have shown that the small signals applied in EIS did not adversely affect the number, viability, and activity of microorganisms within biofilms.

Based on EIS data, Ferrante and Feron concluded that alloy composition of steels was more important for MIC resistance than bacterial population, incubation time, sulfide content, and other products of bacterial growth.[43] Several reports have been published in which EIS has been used to study the role of SRB in corrosion of buried pipes and reinforced concrete. Analysis of these data was qualitative, and no models for impedance behavior in complicated systems have been presented.

Electrochemical Noise Analysis (ENA)

No external signal is applied in ENA; instead, naturally occurring fluctuations of the kinetics of the electrochemical reactions leading to corrosion are monitored. In principle, electrochemical potential or current noise can be recorded in galvanostatic or potentiostatic experiments, respectively. However, evaluations of electrochemical noise phenomena are usually performed at E_{corr}. In early studies, only potential fluctuations were recorded. Potential fluctuations can produce misleading results since usually larger potential fluctuations are observed for passive systems than for actively corroding systems. Current fluctuations are related to the severity of corrosion damage. Therefore, a commonly used approach involves collection of potential and current fluctuations. If potential and current fluctuations are to be converted into the frequency domain and a format similar to impedance data, then current and potential fluctuations

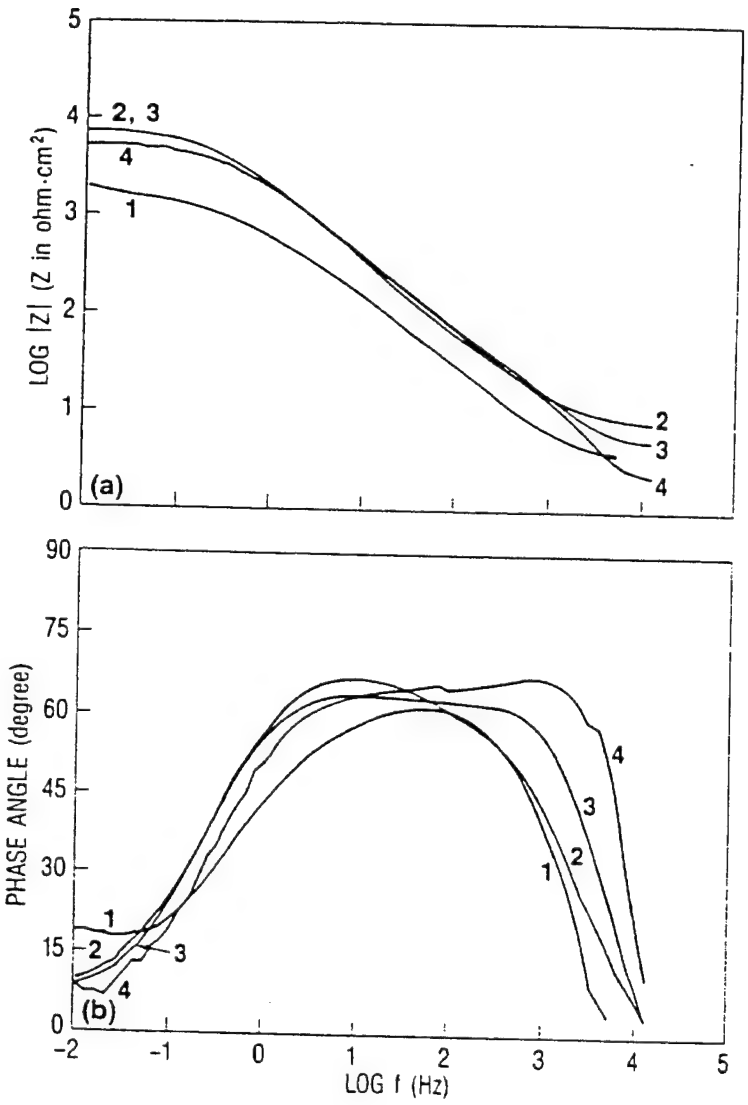


FIGURE 97 - (right) Bode plots for 99copper as a function of exposure time to artificial seawater. Exposure times: 1 day (curve 1); 3 weeks (curve 2); 5 weeks (curve 3); and 8 weeks (curve 4).[30]

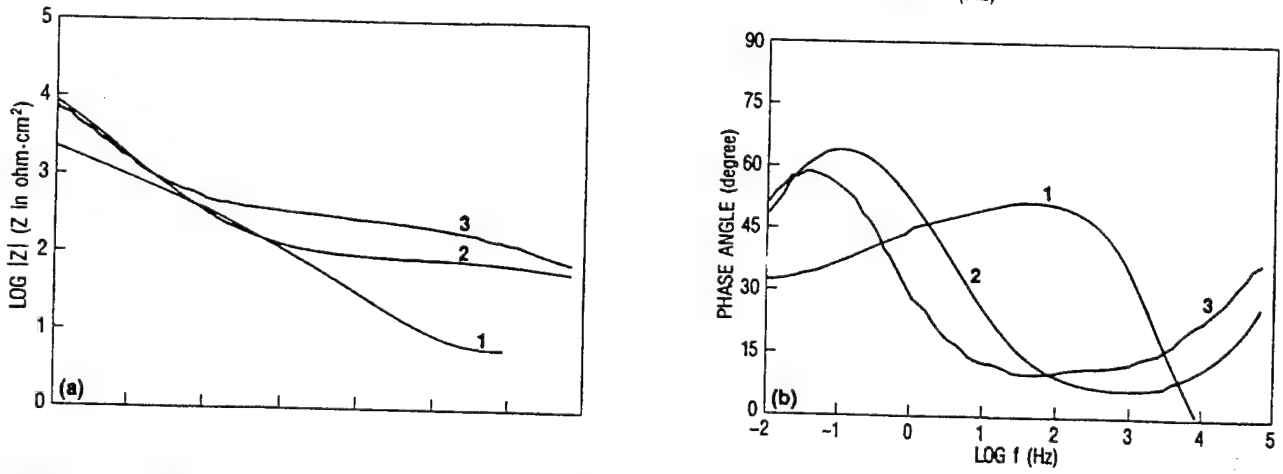


FIGURE 96 - Bode plots for 99copper as a function of exposure time to natural seawater. Exposure times: 1 week (curve 1), 7 weeks (curve 2), and 10 weeks (curve 3).[30]

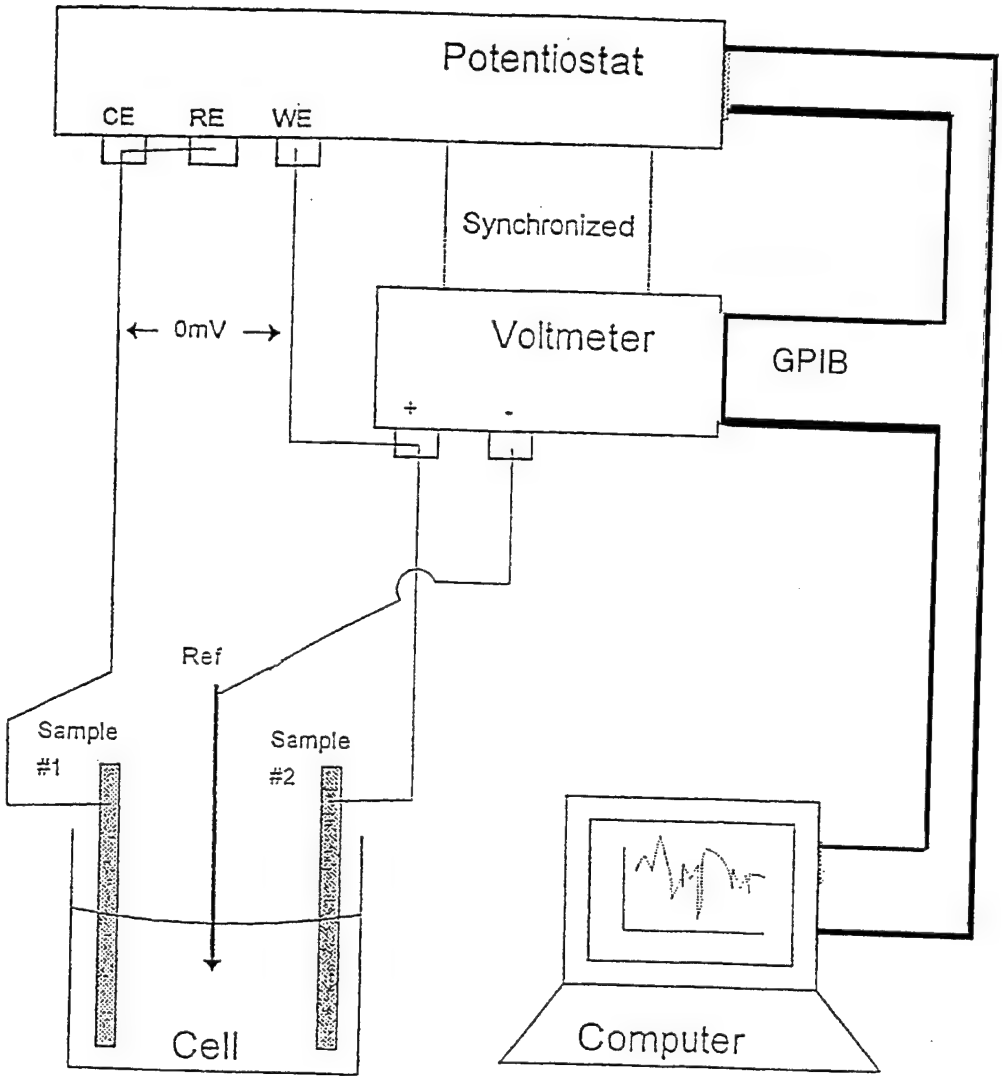


FIGURE 98 - (left) Experimental arrangement for simultaneous collectionof potential, current, and noise data at a remote test site.[44]

must be recorded simultaneously. One reason for recording both potential and current fluctuations is to calculate noise resistance (R_n) defined as:

$$R_n = \sigma\{V(t)\}/\sigma\{I(t)\} \ . \qquad (19)$$

In Equation 19, $\sigma\{V(t)\}$ and $\sigma\{I(t)\}$ are standard deviations of potential and current fluctuations, respectively.

An experimental approach for collecting potential and current fluctuations simultaneously is shown in Figure 98. Two identical electrodes are connected to a ZRA or a potentiostat and galvanic current is monitored as described in Figure 91. The potential of the coupled electrodes is measured vs a reference electrode. It is essential to use suitable software for control of the experiment and data collection of a large number of data points.

In the experimental arrangement shown in Figure 98, the computer controls data collection from the ZRA and a digital voltmeter and stores data. Consideration must be given to frequency of data collection (i.e., number of data/second) and the time for which data are to be collected in each test since these parameters define the accessible frequency range. Electrochemical noise usually occurs below 1 Hz.

Effects of microorganisms on corrosion of concrete

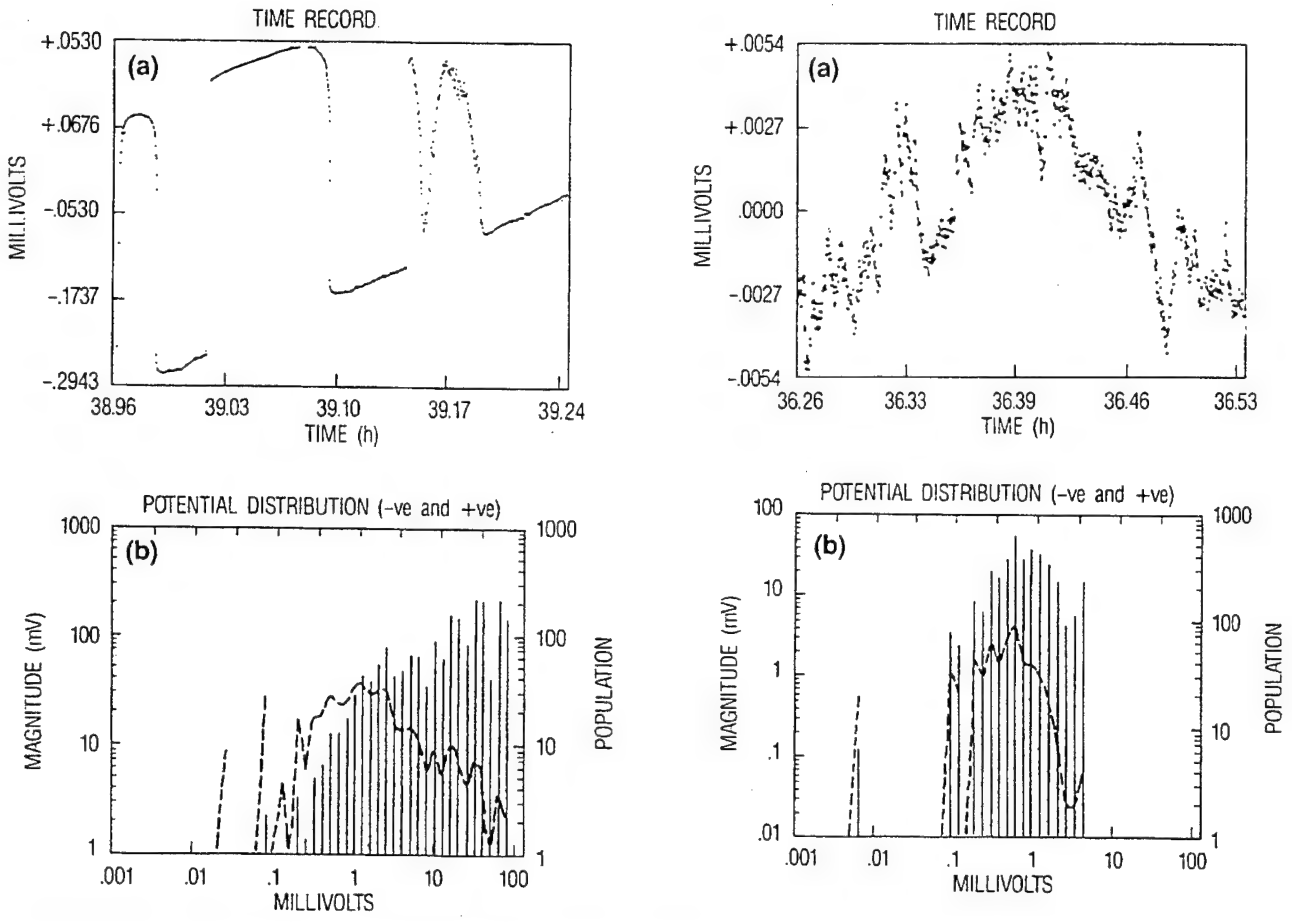


FIGURE 99 - Noise plots, including (a) time records and (b) potential distributions for steel concrete reinforcements after 218 days.[45] (1) covered reinforcements (2) reinforcements exposed to SRB.

and steel reinforcement were studied in an early application of ENA.[45] A portable multichannel instrument was used that also performed zero resistance ammetry. Electrochemical noise data were collected at E_{corr} with a microvoltmeter and a computer. Data were converted into the frequency domain and statistically analyzed. Results for a steel rebar reinforced concrete block exposed to a marine medium containing active SRB compared to results for a covered rebar are presented in two noise plots: a time record and a potential distribution chart showing the population and magnitude of potential fluctuations (Figure 99).

After 218 days, the protective oxide film ruptured on the covered rebar, followed by immediate repassivation. Events recorded for the exposed rebar also showed fluctuations; however, their magnitude was about a factor of 10 lower (Figure 99).

King et al. interpreted noise measurements for steel pipes in environments containing SRB as being indicative of film formation and breakdown.[46] Magnitude of noise fluctuations depends on the total impedance of

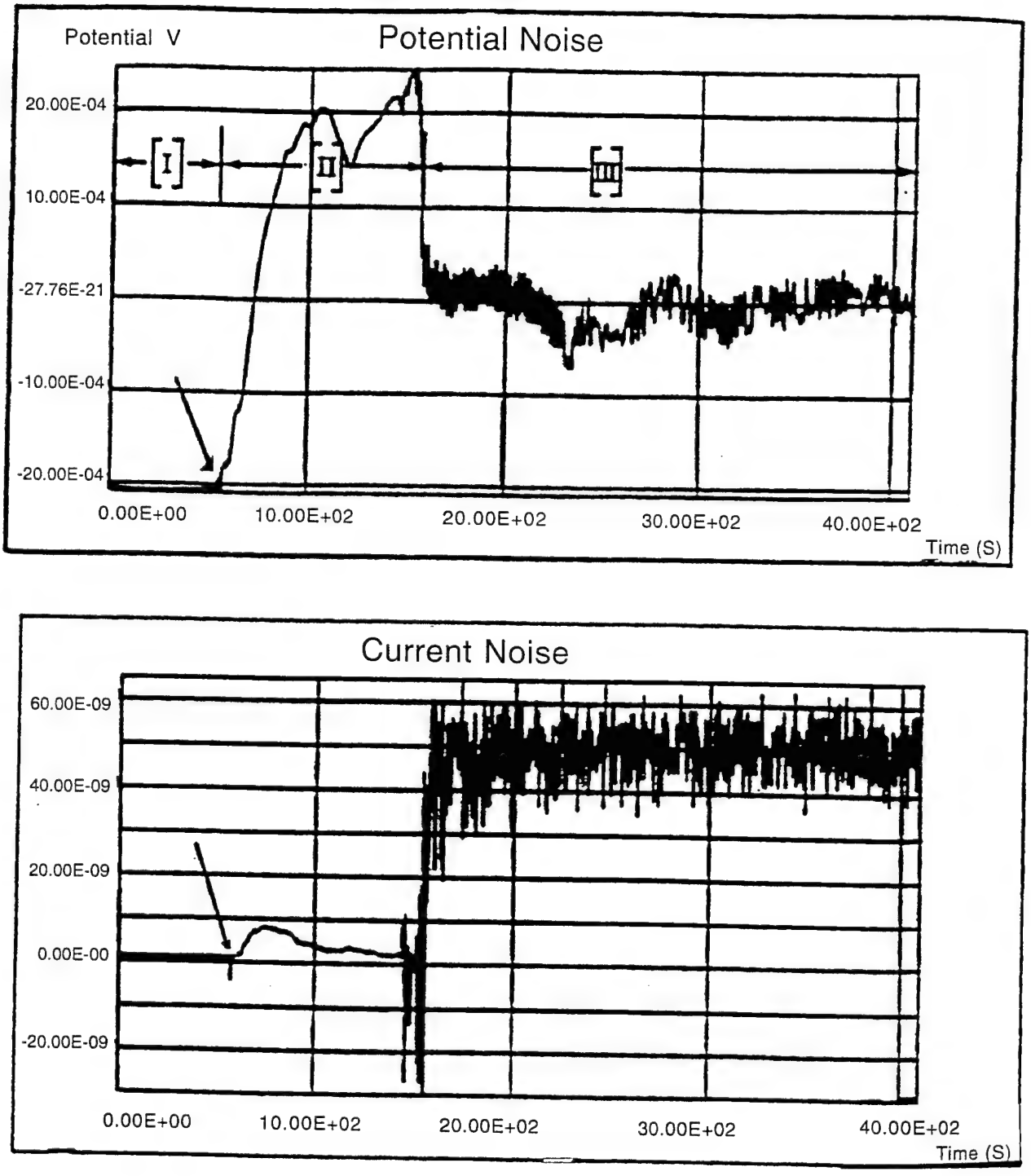


FIGURE 100 - Electrochemical noise data for type 404L stainless steel under stagnant conditions followed by exposure to oxygenated lake water at arrow.[46]

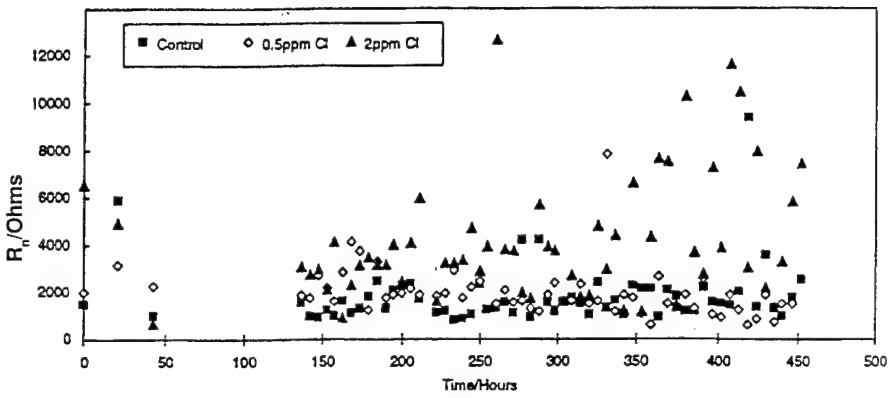


FIGURE 101 - Noise resistance plots vs time for carbon steel, showing effects of hypochlorination at two different concentrations after nine months of exposure.[48]

the system (Figure 100). A corroding metal undergoing uniform corrosion with fairly high corrosion rates may be less noisy than a passive metal with occasional bursts of noise due to localized passivity breakdown followed by rapid repassivation.

Figure 101 shows the time dependence of R_n determined for carbon steel exposed to untreated water after hypochlorite "shock" treatment at two different concentrations. R_n values indicated a beneficial effect of the hypochlorite treatment.

Surface Analytical Techniques

Geesey and Bremer used ATR/FT-IR to evaluate nondestructively, in real time, interactions of bacteria with thin films of copper deposited on germanium.[49] Changes in the thickness of the copper films were measured as increased intensity of the infrared water absorption band at 1,640 cm^{-1}. The authors compared copper loss in the presence of bacteria isolated from corroded copper samples and were able to observe differences between two cultures. Using this technique, Jolley et al. observed copper oxidation by three polymers, including bacterial exopolymer.[50]

Nivens et al. investigated the use of the quartz crystal microbalance (QCM), a very sensitive mass-sensing device, for detecting attached microbial films.[51] The QCM was more sensitive to changes in biomass than ATR/FT-IR, with a detection limit of 10^4 bacteria cm^{-2} and a linear range of at least two orders of magnitude.

An interesting aspect of both ATR/FT-IR and the QCM is that substrata of both techniques can be converted to electrodes for electrochemical analyses so that corrosion information can be obtained while changes in microbial biofilms are monitored.

It is now generally recognized that biofilms alter biofilm/metal interfacial chemistries. Direct chemical measurements are restricted by biofilm thickness and the heterogeneous and anisotropic nature of biofilms.[52] Ion-selective and gas-sensing microprobes with tip diameters less than 10 µm are being used for direct measurements of diffusion-controlled reactions in biofilms. A microprobe 15 µm in diameter was used to simultaneously measure dissolved oxygen and optical density at different depths in a submerged biofilm.[53] The diffusion coefficient for dissolved oxygen, dissolved oxygen flux, and the half velocity coefficient were then calculated. Lewandowski et al. measured dissolved oxygen profiles across a mixed biofilm on a polycarbonate continuous flow, open channel reactor (Figure 102).[52]

Van Houdt et al. developed a rugged iridium oxide pH microelectrode with a tip diameter in the range of 3 to 5 µm.[54] This microelectrode was used to measure a pH profile across a mixed population biofilm on a polycarbonate disk. Lewandowski et al. used an iridium microelectrode to map the spatial distribution of pH over a mild steel surface on which a drop of agar had

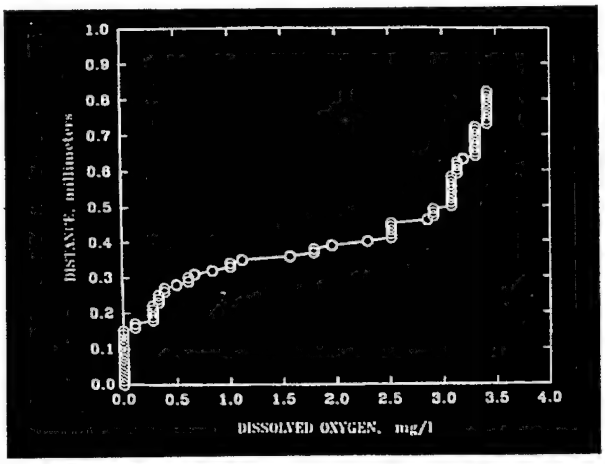


FIGURE 102 - Dissolved oxygen profile across a mixed biofilm using a microelectrode.[52]

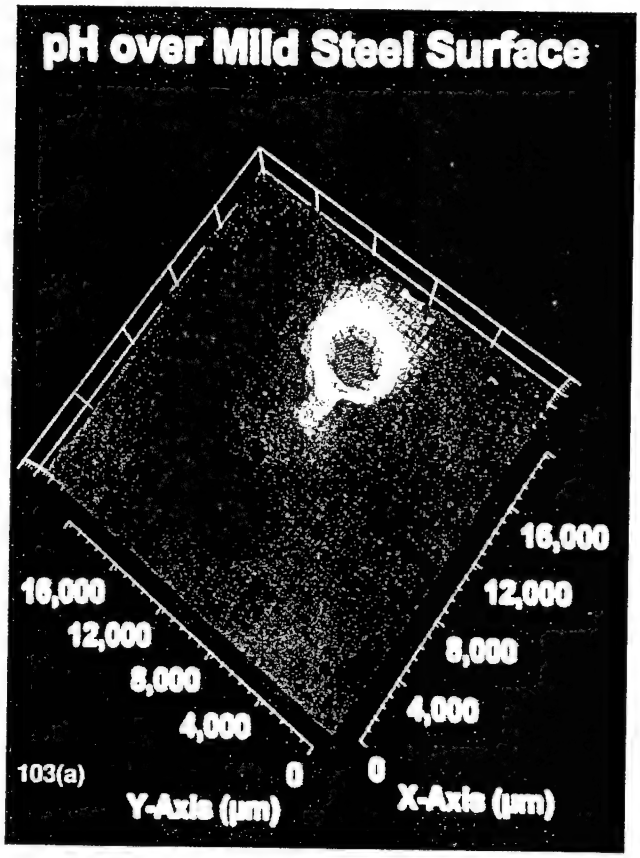
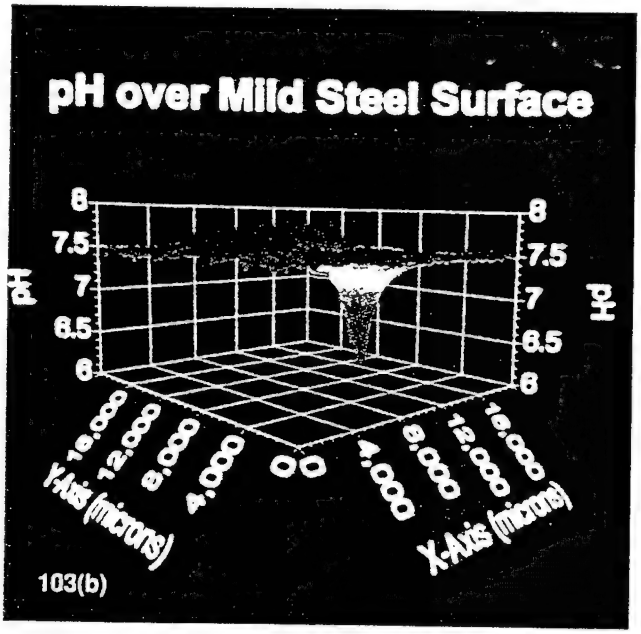


FIGURE 103 - Spatial distribution of pH on a mild steel surface using an iridium oxide microelectrode (a) planar pH distribution on surface partially covered with agar and (b) vertical pH profile of (a).[55]



solidified (Figure 103).[55] The solid polymer created a differential aeration cell. The area immediately under the polymer became anodic. Dissolution of metal and hydrolysis reactions forced the covered area to become acidic.

Recent developments in image analysis systems, including electron, atomic, and laser microscopy, make it possible to image biological materials in the hydrated state. Muellar et al. were able to determine rate coefficients for early bacterial colonization on copper, silicon, stainless steel, and glass using a chemostat, a flow cell, and a microscope equipped with an image analysis system.[56] Substrata were monitored using reflective light from a microscope equipped with a Nomarski lens and microscope output linked to a video camera. Transmitted light was used for transparent surfaces. They demonstrated that surface roughness and surface-free energy correlated positively with biological and abiological sorption processes.

Little et al. used environmental scanning electron microscopy/energy dispersive spectroscopy (ESEM/EDS) to study marine biofilms on stainless steel surfaces.[57] They observed a gelatinous layer in which

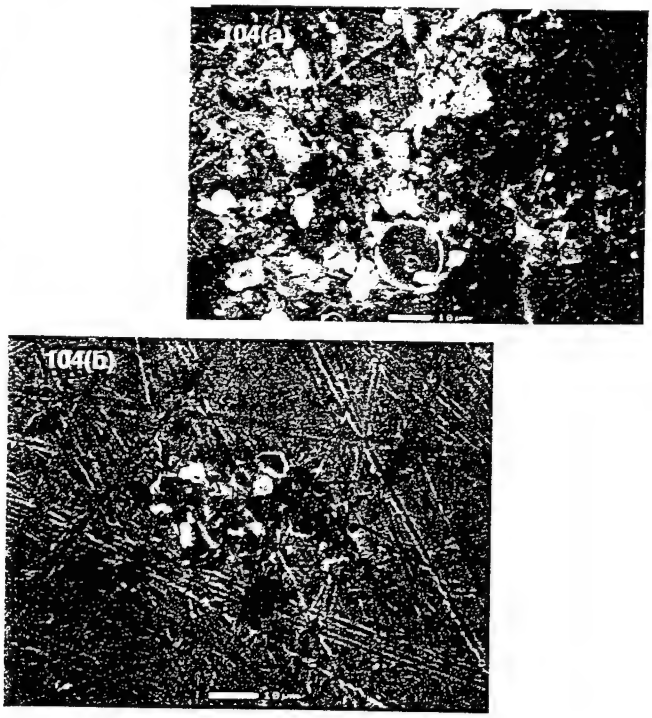


FIGURE 104 - ESEM images comparing areal coverage for (a) wet biofilm directly from water, and (b) same area after acetone/xylene dehydration as for SEM. [57]

TABLE 8.1
WT% OF ELEMENTS FOUND ON STAINLESS STEEL SURFACE AFTER EXPOSURE TO ESTUARINE WATER AND TREATMENT WITH ACETONE AND XYLENE

ELEMENT	BASE METAL	AFTER EXPOSURE TO SEAWATER	AFTER XYLENE
Fe	72.55	11.24	72.43
Cr	18.10	4.71	18.17
Ni	8.02	34.00	7.78
Si	.93	7.35	1.06
Al	.40	3.33	0.57
Cl		0.66	
S		0.99	
K		1.88	
Na		2.12	
Mg		4.98	
Ti		28.74	

bacteria and microalgae were embedded and local concentrations of aluminum, nickel, and titanium. Traditional SEM images of the same areas demonstrated a loss of cellular and extracellular material (Figure 100, page 94).

Dehydration of the biofilm with solvents, required for SEM, either extracted bound metals from the biofilm by ion exchange/solvent extraction or removed the metals with extracellular polymeric material (Table 8.1).

Confocal laser scanning microscopy (CLSM) permits one to create three-dimensional images, determine surface contour in minute detail, and accurately measure critical dimensions by mechanically scanning the object with laser light.[58] A sharply focused image of a single horizontal plane within a specimen is formed while light from out-of-focus areas is repressed from view. The process is repeated again and again at precise intervals on horizontal planes, and the visual data from all images are compiled to create a single multidimensional view of the subject. Geesey used CLSM to produce three-dimensional images of bacteria within scratches, milling lines, and grain boundaries (Figure 105).[59]

The atomic force microscope (AFM) is related to the

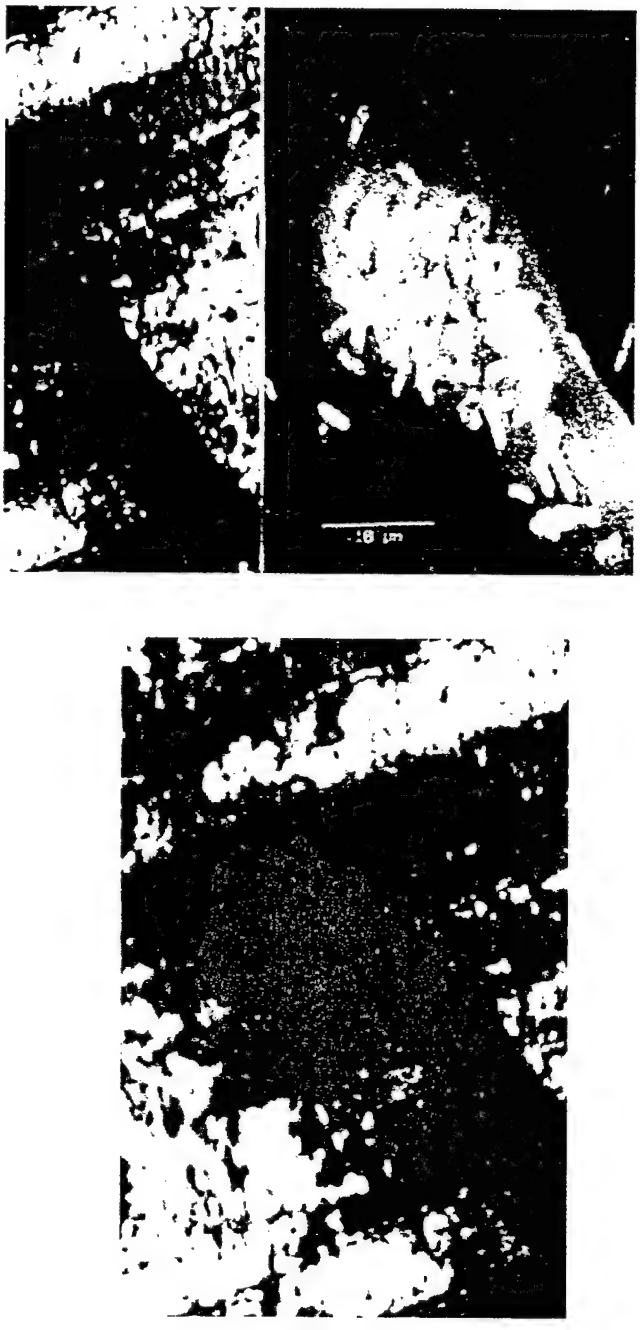
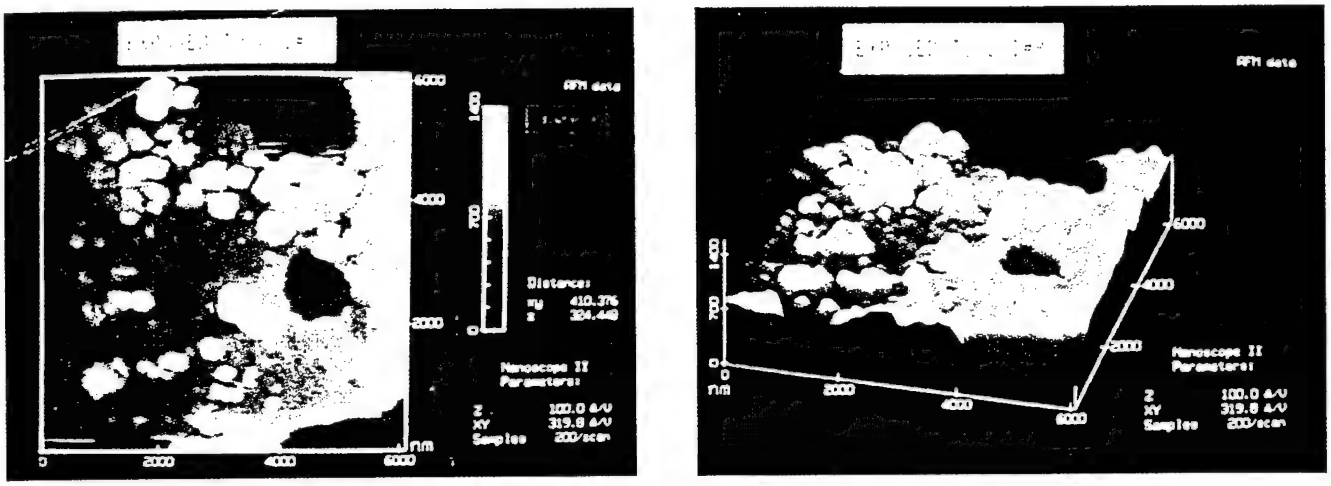


FIGURE 105 - CLSM of a copper surface after several days exposure to a freshwater bacterium (a) reflected visible light image of the surface showing milling lines (dark depressed areas) and high points (light areas), (b) reflected fluorescent light image of same area showing acridine orange-stained cells on surface and (c) fluoresecent image of (b) superimposed on (a) showing localization of cells in milling lines.[59]



Rotated 0° , XY axes (nm), zaxis (nm) Rotated 0° , XY axes (nm), zaxis (nm)

FIGURE 106 - AFM images of hydrated and submerged copper after seven days exposure to bacterial culture (a) biofilm at bulk fluid interface and (b) vertical view of (a).[60]

scanning tunneling microscope (STM). The STM uses an atomically sharp, conductive tip held angstroms from the surface to profile surface features with angstrom resolution. When the tip is electrically biased with respect to the sample, a current flows between the surface atom closest to the tip and the nearest tip atom by the quantum mechanical process of electron tunneling. While the STM requires the sample to be electrically conductive or coated with a conductive material, the AFM can be used to image nonconducting surfaces. The AFM does not rely on tunneling current, provides exceptional detail, and allows viewing of specimens in the hydrated state. AFM uses an extremely sharp scanning probe mounted on a flexible cantilever to record x,y,z coordinates of a sample in fractions of a nanometer. Photodiode electrical outputs mimic sample topography and serve as the basis for the resulting image. AFM images of copper exposed to a bacterial culture for seven days showed biofilms distributed heterogeneously across the surface with regard to both cell numbers and depth (Figure 106). Bacterial cells were reported to be associated with pits on the surface of the copper coupons.

Conversion of metals to sulfides by SRB has been studied since the late 1800s. Baas-Becking and Moore identified mackinawite, greigite, and smythite as indicators

TABLE 8.2
MINERALS FOUND IN CORROSION PRODUCTS ON COPPER EXPOSED TO MIXED FACULTATIVE CULTURES CONTAINING SRB

Bacterial Cultures

	I	II	III	IV	V	VI	VII
99Cu	Chalcocite Digenite	Chalcocite Digenite	Chalcocite Djurleite	Chalcocite Digenite Spionkopite		Chalcocite Digenite	Chalcocite Djurleite
90Cu 10Ni	Chalcocite Djurleite	Chalcocite Covellite Digenite	Chalcocite Spionkopite	Chalcocite Djurleite	Chalcocite Djurleite	Chalcocite Geerite	Chalcocite Djurleite Digenite
70Cu 30Ni						Chalcocite Djurleite Anilite	Chalcocite Djurleite

for SRB corrosion of ferrous metals in anaerobic environments.[61] McNeil et al. analyzed sulfide mineral deposits on copper alloys colonized by SRB in an attempt to identify specific mineralogies indicative of SRB activity (Table 8.2).[62] Bacteria were distributed throughout the corrosion layers associated with the sulfide corrosion products. Some cells were encrusted with sulfide deposits (Figure 107). The authors concluded that formation of nonadherent layers of chalcocite (Cu_2S) and the presence of hexagonal chalcocite were indicators of SRB-induced corrosion of copper. The compounds were not observed abiotically, and their presence in near-surface environments could not be explained thermodynamically.

Sulfur isotope fractionation was demonstrated by Little et al.[63] in sulfide corrosion deposits resulting from activities of SRB within biofilms on copper surfaces (Table 8.3). ^{32}S accumulated in sulfide-rich corrosion products. ^{34}S was concentrated in the residual sulfate in the culture medium. Accumulation of the lighter isotope was related to surface derivatization or corrosion as measured by weight loss. Use of this and the preceding mineralogical technique to identify SRB-related corrosion requires sophisticated laboratory procedures.

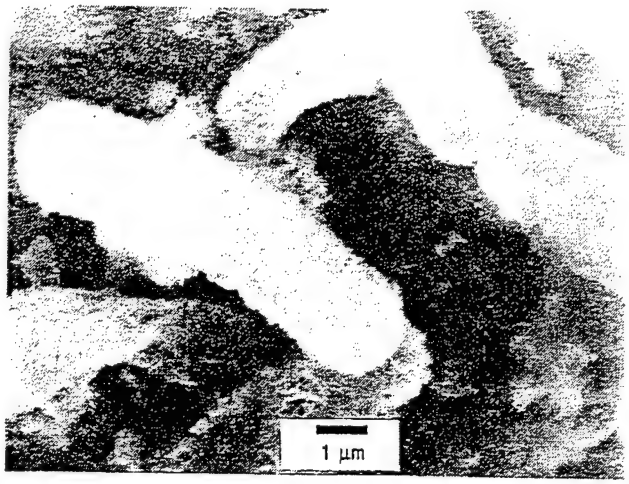


FIGURE 107 - ESEM image of sulfide deposits on cells exposed to a mixed facultative culture containing SRB.[62]

TABLE 8.3
SULFUR ISOTOPE AND CORROSION DATA AFTER 3 MONTHS EXPOSURE TO MIXED FACULTATIVE CULTURES CONTAINING SRB

Sample	Sulfur Concentration in Solid ppm	$\delta\ ^{34}S$ in Solid ‰	Sulfur Concentration in Medium ppm	$\delta\ ^{34}S$ in Medium ‰	Weight Loss $mg/mm^2 \times 10^{-3}$ for Similarly Treated Foils
Control	747	10.2	372	0.3	15.9
	900	12.3	514	0.4	30.2
	613	16.5	580	0.2	29.9
SRB Culture I	26627	−0.4	7	*	226.3
	36618	−1.3	20	3.7	131.7
	36695	−0.8	16	8.7	178.6
SRB Culture II	4321	0.6	20	23.8	99.4
	3371	1.5	18	25.8	227.2
	19490	−0.7	11	*	169.5

*Insufficient sulfur

Conclusions

Failure Analyses

There can be only one definitive test for MIC in a failure analysis. It involves isolation of the causative organisms from the corrosion site and a demonstration that (a) the organisms can reproduce the phenomenon in an unaffected metal or alloy and (b) the effect is not present in an environment that is identical except for the absence of the organisms.

Such demonstrations have been extremely rare because they require long periods of time. Furthermore, it is difficult to reproduce the exact environmental conditions of the original corrosion phenomenon and to maintain microorganisms in their natural consortia. Instead, a spatial relationship is typically established between microorganisms known to contribute to corrosion and the localized attack.

Test kits or direct culture techniques should be used to determine the numbers and types of cells. Microscopy, metallurgy, and surface chemistry should be used to relate microorganisms to the localized corrosion.

It is important not only to document high numbers of organisms, but also to identify specific types of organisms that can be linked to an apparent corrosion mechanism. Finding high numbers of SRB plus metal sulfides certainly makes a strong case for MIC. The presence of *Gallionella* in conjunction with subsurface cavities along stainless steel welds and deposits containing high levels of iron, manganese, and chlorine makes a strong case for MIC of stainless steel.

Monitoring

In industrial situations, one must be able to access vulnerable systems with side streams, coupons, or probes to collect microbiological and electrochemical data. Measurements of E_{corr} and redox potential can be used to determine changes in the electrolyte at the metal surface, but cannot be used independently or together to determine corrosion rates. Techniques that incorporate zero resistance ammeters can be used to detect the onset and extent of MIC. Electrochemical current noise and potential noise perturbations can signal the onset of localized corrosion. R_n determined from electrochemical potential noise/electrochemical current noise data is considered equivalent to R_p. Both types of tests must be accompanied by some measure of microorganisms or their activity to specify MIC.

Prediction

Data used to make predictions about the propensity of metal/microbe combinations to produce MIC are routinely generated in the laboratory and often involve zero resistance ammetry or large signal polarization techniques with abiotic controls. In these types of experiments, microorganisms have been selected or characterized prior to the initiation of the experiment. Their types, numbers, and spatial relationship to corrosion should be verified at the end of the experiment. All techniques discussed in Chapter 8, "Recent Laboratory Developments," have applicability in the prediction of MIC.

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Resource Addresses

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Angus Chemical Co., 2211 Sanders Rd., Northbrook, IL 60062

ASTM, 100 Barr Harbor Dr., West Conshohocken, PA 19428

Bioindustrial Technologies, Inc. 40200 Industrial Park Circle, Georgetown, TX 78626

Biosan Laboratories, 10657 Galaxie, Ferndale, MI 48220

Caproco Ltd., 8741-53 Ave., Edmonton, Alberta, Canada T6E 5E9

Conoco Specialty Products, 600 N. Dairy Ashford, Houston, TX 77079

E.I. du Pont de Nemours & Co Inc, Wilmington, DE 19898

Gen-Probe Inc., San Diego, CA 92121

NACE International, 1440 South Creek Drive, Houston, TX 77084

Petrolite Co., 369 Marshall Ave., St. Louis, MO 63119

Williams Brothers (Laboratories of) Engineering Co., 6600 S. Yale Ave., Tulsa, OK 74136

Glossary

Aerobe - an organism that grows in the presence of oxygen. Growth may occur only in the presence of oxygen (obligate) or may occur in the absence of oxygen (facultative).

Algae - small single or simple multicellular plant-like organisms which grow normally in the presence of light by photosynthesis. Occur widely in freshwater and are related in population size to the degree of nutrients available to the water body. Some are capable of using organic materials for growth (heterotrophy) and may occur in wide bore or shallow wells and in deeper wells, where there is an adequate organic nutrient base.

Anaerobe - an organism that can grow in the absence of oxygen. Many of these bacteria are known to be so sensitive to oxygen that the presence of even low levels (i.e., 0.1 mg O_2 /L^{-1}) will kill the cells.

Anode - a positively charged electrode.

Antibody - protein generated in response to foreign protein or polysaccharide.

Autoclave - A pressurized, steam-heated vessel used for sterilization.

Biocides - specific compounds or groups of compounds which can, in either a gaseous, dry, suspended or dissolved state, cause a toxic (lethal) effect on a broad spectrum of target organisms.

Biodeterioration - biologically mediated deterioration or destruction of materials, such as paper products, woods, concretes, paints, and metals.

Biofilm - microbial growth on a surface in which individual cells are bound within a matrix of extracellular polymeric materials.

Biofouling - deterioration of any type resulting from biological activities.

Bioluminescence - emission of visible light by living organisms.

Biomass - total mass of biological material within a given system usually estimated as a dry weight.

Cathode - a negatively charged electrode.

Colony - a population of cells which grow as a cluster or assemblage on a solid surface sufficiently to become visible. Common surfaces used are agar gels or membrane filters.

Colony-forming unit (cfu) - one viable cell that will form a separate colony.

Community structure - microbial relationships/interdependence within a biofilm.

Consortium - group of cooperative organisms that maintain a common econiche.

Corrosion - deterioration of solid or porous materials and their surfaces by physical and/or chemical erosive mechanisms which can be influenced by the presence of microorganisms.

Culture - (n.) observable growth of specific microorganisms in laboratory media; (v.) to grow specific microorganisms in laboratory media.

Diluent - a substance used to dilute.

Dissimilatory - metabolic changes that convert complex molecules to simple ones.

Electrolyte - a substance that dissociates into ions in solution and becomes a conductor.

Enzyme - protein produced by a living organism that functions as a catalyst.

Facultative - indicative of an organism capable of performing in the presence or absence of the environmental factor being defined.

Filamentous - slender and threadlike.

Fluorochrome - fluorescent dye used to stain microorganisms for examination by fluorescence microscopy.

Fungus - plants lacking chlorophyll, ranging in form from a single cell to a body of branched filamentous hyphae. Group includes yeasts, molds, smuts, and mushrooms.

Galactoside - any of a group of organic compounds that produce galactose on hydrolysis.

Gallionella - a gram negative vibrioid to straight rod cell which excretes a ribbon-like twisted stalk out of one side of the cell, widely believed to be the dominant genus in iron-related bacteria consortia.

Gene - a functional hereditary unit that occupies a fixed location on a chromosome, has a specific influence on phenotype, and is capable of undergoing mutation.

Heterotrophic bacteria - bacteria which use organic materials as principal sources of energy and carbon for survival, growth, and synthesis.

Hydrolysis - chemical decomposition involving bond splitting and addition of water.

Immunoassay - analysis and identification of a substance based on antigenic reactions.

Iron-oxidizing bacteria - bacteria capable of oxidizing ferrous iron to the ferric form, commonly an aerobic event.

Iron-precipitating (depositing) bacteria - bacteria which force the precipitation of iron salts inside or around the cell or within the aquatic environment.

Iron-reducing bacteria - bacteria capable of reducing ferric iron to the ferrous form, commonly an anaerobic event.

Iron-related bacteria (IRB) - bacteria that accumulate iron. Growths tend to be predominantly orange-red to brown. Any bacterium that can use iron within its life cycle in a defined manner whereby the oxides and hydroxides become either bound within or exterior to the cell.

Isotope - one of two or more atoms whose nuclei have the same number of protons but different numbers of neutrons.

Microflora - microorganisms.

Most probable number (MPN) - a statistical method for the prediction of a microbial population in a liquid by the presence/absence of growth in various dilutions.

Negative staining - a staining technique in which the dye forms a dark background while the specimen remains unstained.

Non-viable - not capable of living.

Obligate - descriptive of environmental factors essential to the survival, growth, and reproduction of an organism.

Oligotroph(ic) - an organism able to flourish in an environment having a low-nutrient regime.

Pasteurization - the process of heating to a sufficient extent to reduce the number of nuisance microorganisms to an acceptable and manageable level. Usually performed within strict temperature and time constraints (e.g., 60°C for 30 minutes).

Periphyton - organisms that live attached to surfaces in aquatic environments.

Petri dish - a shallow glass or plastic vessel with a fitted lid that allows microorganisms to be grown under aseptic (free from viable contaminants) conditions usually in agar culture media.

Plankton - microscopic organisms that float or drift in water.

Recolonization - re-establishment of a microbial population.

Ringer's solution - an aqueous solution of chlorides that is isotonic to animal tissue.

Sessile - growing on a surface.

Spreadplate - a technique where the bacteria to be enumerated are spread out over the surface of a suitable agar culture medium.

Thermophilic - requiring high temperature for growth and development.

Tubercles - raised encrustations on a metal surface.

Viable - capable of living under favorable conditions.

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